

FUNGAL ANTAGONISTS OF THE SOYBEAN CYST NEMATODE
HETERODERA GLYCINES

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The present work is dedicated to the memory of my grandfather, Yanhuan Chen.

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FUNGAL ANTAGONISTS OF THE SOYBEAN
CYST NEMATODE *HETERODERA GLYCINES*

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Chairperson: D. W. Dickson
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The mycoflorae in females and cysts of *Heterodera glycines* were investigated in a Florida soybean (*Glycine max*) field, and their influences on the nematode were estimated in the laboratory and greenhouse. In the field, the frequency of fungi colonizing white and yellow females was low, but a high frequency of fungi was encountered in brown cysts and the frequency increased with time of exposure of the cysts to the soil. The common fungi isolated from the females and cysts were *Neocosmospora vasinfecta*, *Fusarium solani*, *Fusarium oxysporum*, *Dictyochaeta coffeae*, *Dictyochaeta heteroderae*, *Pyrenochaeta terrestris*, *Exophiala pisciphila*, *Gliocladium catenulatum*, *Stagonospora heteroderae*, and a black, yeast-like fungus.

In a greenhouse study, the percentages of cysts colonized and eggs parasitized by fungi in soil from five sites treated with microwave heating were lower than in untreated soil. The nematode densities were higher in treated soil than untreated soil. The

nematode density was correlated negatively with fungal colonization of cysts and eggs. No differences in nematode densities and frequencies of fungal colonization of cysts or eggs in soil were observed, however, among the five sites.

Twenty-one isolates of 18 species of fungi were tested on water agar for their pathogenicity to eggs. *Verticillium chlamydosporium*, *P. terrestris*, and two sterile fungi parasitized a relatively high percentage of eggs and reduced hatching rates 42-73%. *Arthrobotrys dactyloides*, *F. oxysporum*, *Paecilomyces lilacinus*, *S. heteroderae*, *N. vasinfecta*, *F. solani*, and *E. pisciphila* were moderately pathogenic to eggs. *Beauveria bassiana*, *Hirsutella rhossiliensis*, *Hirsutella thompsonii*, *D. heteroderae*, *D. coffeae*, *G. catenulatum*, and *Cladosporium* sp. showed low or no parasitism of eggs, but reduced the hatching rate. The hatching rate was correlated negatively with the percentage of eggs parasitized. In a greenhouse study, nematode densities in soil treated with *H. rhossiliensis* or *V. chlamydosporium* were lower than that in untreated soil. Light, scanning electron, and transmission electron microscopy studies revealed that *A. dactyloides*, *Dictyochaeta heteroderae*, *E. pisciphila*, *F. oxysporum*, *N. vasinfecta*, *Paecilomyces lilacinus*, *P. terrestris*, *S. heteroderae*, *Verticillium chlamydosporium*, and the black, yeast-like fungus penetrated the cyst wall. At least three species, *E. pisciphila*, *F. oxysporum*, and *P. terrestris* penetrated through the cyst wall from the outside.

CHAPTER 1 INTRODUCTION

Fungal Antagonists of Nematodes

Historical Background

Fungal antagonists of nematodes have been studied since the first observation of the nematophagous habit of the fungus, *Harposporium anguillulae* Zopf, by Lohde²⁸¹ in 1874. Linford's^{274,277} efforts to use predacious fungi to control plant-parasitic nematodes stimulated interest in the nematode-trapping fungi. Early researches done in France, the United States, England, and the former USSR were substantial contributions to our understanding of the taxonomy and ecology of the nematode-trapping fungi, but there has been only limited success using them as biological control agents against nematodes.⁴⁴⁸ The lack of success of these early experiments resulted in a decline in interest in biological control of nematodes. The resurgence of interest in biological control occurred in the mid-1970s. This resulted from both the continuing environmental problems associated with the use of nematicides^{239,241,448,467} and the demonstration of suppression of the cereal cyst nematode, *Heterodera avenae* Woll.,^{245,247,506} and *Meloidogyne* spp.^{222,450,453} by fungal parasites. Some efforts have been made to market biological control agents^{4,49,469} for nematode management, but the products generally have not been accepted. Biological control of nematodes with fungal antagonists " is in the

invidious position of needing a successful example of the contrived use of an antagonist to demonstrate to administrators and funding agencies that further work is worthy of support.⁴⁴⁷ⁿ

Types of Fungal Antagonists of Nematodes

Fungal antagonists of nematodes are those fungi, including nematophagous and non-nematophagous fungi, that have some adverse effect on nematodes. To date, more than 350 species of fungi have been isolated from nematodes.^{67,394} Cooke and Godfrey⁶⁷ included 97 predaceous and endoparasitic fungal species attacking vermiform nematodes in a key. Crump⁷⁵ listed 129 species of fungi isolated from beet, cereal, and potato cyst nematodes. Rodríguez-Kábana and Morgan-Jones³⁹⁴ summarized fungal species isolated from root-knot and cyst nematodes.

Nematophagous fungi use nematodes for nutrition. In early literature nematophagous fungi were referred to as predacious and endoparasitic fungi. After accumulation of knowledge of fungal parasites of eggs, females and cysts of sedentary endoparasitic nematodes, such as Heteroderidae, a third group of nematophagous fungi was proposed: opportunistic fungi.³³¹ In this review, however, the fungal antagonists of nematodes are grouped into (i) predacious fungi, (ii) obligate endoparasitic fungi, (iii) facultative endoparasitic fungi, (iv) fungi producing antibiotic substances, and (v) vesicular-arbuscular (VAM) mycorrhizal fungi and other endophytes.

Predaceous fungi

Predaceous organisms capture, kill, and then consume their prey. Like some

omnivorous plants, the predaceous fungi have evolved special devices for capturing animals such as nematodes. These devices are adhesive hyphae, adhesive branches, adhesive nets, adhesive knobs, nonconstricting rings, and constricting rings.^{18,67,122,176} However, killing of nematodes by some predaceous fungi may be a slow process and fungi may undergo parasitism for a long period. Based on this point of view, these fungi are considered parasites of nematodes.

Fungi with adhesive hyphae. Adhesive hyphae are usually produced by lower fungi, such as Zygomycetes, which are usually in the genera *Stylopaga* and *Cystopaga*. Because they have no septa, they can not produce complex devices for capturing nematodes.¹⁸ However, septate fungi in Deuteromycetes, such as *Arthrobotrys botryospora* Barron,²⁰ *Dactylaria psychrophila* Drechsler,¹⁷⁴ and *Arthrobotrys superba* Corda,²¹⁷ capture nematodes with adhesive hyphae. These fungi produce adhesive materials that are deposited on some points of hyphal surfaces. When a nematode touches these points it is captured. The hyphae produce appressoria that penetrate through the nematode body wall and the mycelium grows throughout the nematode body. After consuming all of the nematode contents, the fungus draws all plasma back outside of the nematode body for the development of sporangia and spores.

Fungi with adhesive branches. Adhesive branches are produced by Deuteromycetes and lower fungi such as *Stylopaga*. The behavior of these fungi is similar to that of adhesive hyphae. Adhesive branches are usually made up of one to three cells and normally anastomose to form simple adhesive hoops or two dimensional networks. A thin film of adhesive materials is secreted over the entire surface of the

branch. The trap is elevated above the substrate. Nematodes that become attached to branches are held fast. They are quickly penetrated and consumed. The adhesive branches are probably a primitive trap from which more complex organs of capture have evolved.¹⁸

Fungi with adhesive nets. Adhesive nets are common trapping devices of nematode-trapping fungi.⁶⁷ Adhesive nets are usually found in the Deuteromycetes. *Arthrobotrys oligospora* Fresenius is probably the most commonly encountered of all the predaceous fungi.¹³⁴ It has been studied extensively to determine its mechanism of infection of nematodes and its efficacy in reducing nematode densities. Nordbring-Hertz³⁵⁸ demonstrated the presence of an adhesive substance coating the surface of the capture organs. The adhesive nets are effective and once a nematode is captured, the prey is held fast. If the nematode struggles, it becomes attached to another net and may become attached to several nets.^{18,176} Large nematodes as well as small nematodes can be captured by this device. Once a nematode is captured, a penetration hypha enters the nematode within 1 hour and then swells to form an infection bulb.⁴²⁶ From this structure assimilative hyphae develop.

Fungi with adhesive knobs. Adhesive knobs are morphologically distinct cells and they are considered to be highly specialized trapping devices. They are covered by a thin layer of adhesive materials. They are either sessile or borne at the apex of a short nonadhesive stalk. When a nematode touches the knobs, a flattened mass of adhesive is produced at the point of attachment, forming a thick pad. This increases the area of attachment many fold, ensuring that the nematode is firmly held.^{110,111,176} Adhesive knobs

contain numerous electron-dense spherical bodies close to that part of the cell wall likely to come into contact with a nematode.^{507,508} Adhesive knobs are normally quite closely spaced along hyphae, and when a nematode is caught it is quite normal for it to become attached to several other knobs as it struggles to free itself, making escape impossible.¹⁷⁶ In the struggle to escape, the nematodes usually break the knob off and carry the knob on their body surface.¹⁷ The knob then penetrates the host and the nematode is parasitized. Penetration by fungi may result from a mixture of chemical¹⁴¹⁸ and physical activities.¹⁷⁶ The adhesive knobs are found in Deuteromycetes and Basidiomycetes. Deuteromycetes, such as *Dactylaria candida* (Nees) de Hoog & van Oorschot, produce a globose infection bulb after penetration. From this bulb, the assimilative hyphae arise to colonize and digest the host contents. Basidiomycetes, such as *Nematoctonus* sp., do not form an infection bulb after penetration. This group of fungi is unique in forming the clamp connections on the secondary hyphae.

Fungi with nonconstricting rings. Nonconstricting rings are produced by erect, lateral branches arising from the prostrate septate hyphae of Deuteromycetes, such as *Dactylaria*. Fungi that produce nonconstricting rings, as exemplified by *D. candida* and *Dactylaria lysisipaga* Drechsler, often produce adhesive knobs as well.¹¹³ Knobs and rings are commonly found alternating on the same hyphal strand.¹⁷⁶ Also, an adhesive layer, similar to those associated with adhesive nets and adhesive knob traps, has been observed on the inside of the ring.^{111,112} The ring is composed of three cells. When a nematode enters the ring and if its diameter is only a little larger than the inner diameter of the ring, the ring will hold the nematode. The connection between the ring and stalk is very

delicate and the nematode usually will break off the ring in its struggle to escape. One nematode may carry several rings around its body. Finally the fungus penetrates the nematode body wall and consumes the nematode.

Fungi with constricting rings. The most sophisticated device for trapping fungi is the constricting ring formed by species of *Dactylella*, *Dactylaria*, *Arthrobotrys*, and a few other Deuteromycetes. The constricting rings, which also consist of three cells, are similar to nonconstricting rings in morphology. However, unlike the nonconstricting ring, the constricting ring is borne on a strong stalk that is not easily broken. The size of the ring varies between and within species, but generally falls within a range of 20 to 40 μm .¹⁷⁶ When the constricting ring is stimulated, the three cells expand rapidly inward. Closure of the ring takes place in about 0.1 second.¹⁷⁶ If a nematode moves into the ring, the inner walls of the ring cells will be triggered to close and the nematode will be pinched and have no chance to escape. The mechanisms involved in closure of the rings, however, are debatable. Both mechanical and hot water, but not chemical stimulation, have triggered the swelling of the constricting rings.^{71,342} Couch⁷¹ believed that the closure was affected by swelling of a colloidal substance. The swelling may be due to a rearrangement of molecules of water and colloidal material already in the cell, or it might be that additional water is imbibed from the stalk and thread cells. Muller³⁴² argued that colloidal material could not account for ring closure. Muller³⁴² suggested that stimulation of the inner walls of the three cells changed the permeability of the cell membranes and the cells absorb water from the surrounding medium.

Obligate endoparasitic fungi

The obligate endoparasitic fungi are different from the trapping fungi in that they have no special trapping devices and they have no extensive hyphal development outside the body of nematodes.¹⁸ The obligate endoparasitic fungi attack either vermiform nematodes, nematode eggs, or sedentary females.

Obligate endoparasites of vermiform nematodes. The obligate endoparasitic fungi that attack vermiform nematodes can be found in lower and higher fungi. They can be placed into three groups based on their mechanism of infection: 1) encysting species, 2) species forming adhesive conidia, and 3) species with conidia that may be ingested.^{18,176}

Encysting species: Encysting species that produce zoospores belong to Chytridiomycetes and Oomycetes. The zoospores can swarm for a short time and encyst on nematode cuticles if they reach their host nematode. *Catenaria* spp., which belong to Chytridiomycetes, are the most common endoparasitic fungi encountered on vermiform nematodes. Their zoospores have one posterior whiplash flagellum and sporangia are formed in chains within the nematode body. *Myzocytiium* spp. (Oomycetes), which also can parasitize nematodes, are different from *Catenaria* spp. in that they produce oospores and zoospores with two flagella, one tinsel and the other whiplash.

Species forming adhesive conidia: The endoparasitic fungi forming adhesive conidia are found in Zygomycetes, Basidiomycetes, and Deuteromycetes. Species of *Meristacrum* (Zygomycetes) form conidia that are forcibly discharged at their maturity. If the primary conidia fail to contact a nematode, they may germinate and produce a secondary conidium. Both primary and secondary conidia have a layer of sticky material

on their surfaces. The adhesive conidia formed by species of Deuteromycetes are streamline in shape and adhesive only at one end, at which an adhesive bud is formed. The endoparasitic basidiomycetes also infect nematodes with adhesive conidia, but the conidia form adhesive knobs similar to those formed on hyphae by the predaceous species.

Species with conidia that may be ingested: Some species of endoparasites have developed morphologically adapted conidia, which, when eaten by a nematode, become lodged in either its buccal cavity or esophagus. The species belong almost exclusively to the genus *Harposporium* (Deuteromycetes). The conidia are crescent-shaped, helicoid, or irregular-shaped, and they are well adapted to infection. Plant-parasitic nematodes can not be infected by these fungi because the lumen of the stylet is too small to ingest the conidia.

Obligate endoparasites of eggs and sedentary females. Obligate parasites of nematode eggs and sedentary females cannot develop mycelium in soil. Therefore, these fungi must have a motile or disseminative stage in order to reach the target nematode, because eggs or females cannot move toward the fungi. Three species, *Catenaria auxiliaris* (Kühn) Tribe, *Nematophthora gynophila* Kerry & Crump, and an undescribed lagenidiaceous fungus, have been reported on cyst-nematodes.^{237,244} All of these fungi produce thick-walled resting spores that remain in soil from one season to the next. The zoospores are probably the infective stage.^{448,474} Zoospores move toward nematode females. When they contact the target nematode, they penetrate the body wall and assimilate the nematode's body contents. *Catenaria auxiliaris* and *N. gynophila* also

attack nematode eggs within the female body.^{231,474} *Catenaria auxiliaris* is encountered frequently on *Heterodera schachtii* Schmidt^{466,474} and *H. avenae*^{232,243} in Europe. It also has been found on *Heterodera glycines* Ichinohe in the United States⁸³ and China,²⁷⁸ and on *H. avenae* in Australia.⁴⁴⁹ *Nematophthora gynophila* is common on *H. avenae* and *H. schachtii* in Europe^{237,244} and it also has been found on *H. glycines* in the United States.⁸³ An isolate of *Nematophthora* was reported on *H. glycines* in China that differs from *N. gynophila* in size of oospores.²⁷⁸

Facultative endoparasitic fungi

Facultative endoparasitic fungi are those fungi that can develop in soil without existence of nematodes and are usually called opportunistic fungi.

Facultative parasites of eggs and sedentary females. Facultative parasites are usually associated with eggs, egg masses, females, or cysts of Heteroderidae. These fungi produce an expanding mycelium growth that enables them to reach the nematode targets of sessile stages. These fungi belong to taxonomic divergent groups of Chytridiomycetes, Basidiomycetes, and Deuteromycetes, but they are encountered more frequently in Deuteromycetes.

Facultative endoparasites of vermiform nematodes. Most fungal endoparasites of vermiform nematodes are obligate parasites.⁴⁴⁸ There are a few fungi, such as the species *C. anguillulae*, that can grow saprophytically on dead or injured nematodes.⁴¹⁶ *Hirsutella rhossiliensis* Minter & Brady is considered an obligate parasite.^{205,241} It can be cultured on various substrates; therefore, it is probably appropriate to group this fungus with facultative parasites. Some trapping fungi, such as those with adhesive

knobs, act very similarly to these endoparasitic fungi that form adhesive conidia. Consequently, the trapping fungi with adhesive knobs can be considered facultative endoparasites of vermiform nematodes. Many fungi, such as *Verticillium chlamydosporium* Goddard, that colonize cysts or egg masses also can attack second-stage juveniles within the cysts or egg masses. Although the mode of infection of juveniles in cysts or eggs masses is poorly understood, these fungi are probably facultative endoparasites of the vermiform juveniles.

Fungi producing antibiotic substances

Many fungi isolated from cysts and egg masses are probably saprophytic. Their effects on nematodes are not clear. Presumably, some of these fungi produce substances toxic to nematodes,^{48,236,330} or their existence in egg masses or cysts inhibits hatching of juveniles from eggs. Culture broth of *Paecilomyces* spp. and *V. chlamydosporium* suppressed hatching of *Meloidogyne incognita* (Kofoed & White) Chitwood.⁴¹ A similar effect of culture filtrate of *Paecilomyces lilacinus* (Thom) Samson on hatching of *M. incognita* was observed by Khan et al.²⁵³ Culture filtrates of *A. oligospora*, and *Dactylaria brochopapa* Drechsler inhibited hatching of *M. incognita*, while *Curvularia pallescens* Boedijn inhibited hatching of *Heterodera zaei* Koshy, Swarup & Sethi.²⁶⁸ Culture filtrate of *Aspergillus niger* van Tiegh, a nonpathogenic fungus, killed 93% of *Meloidogyne* sp. juveniles even when the culture filtrate was diluted 20 times.⁹² *Paecilomyces lilacinus* releases chitinase and protease which can cause undifferentiated *Meloidogyne hapla* Chitwood eggs to become deformed and vacuolate.¹³⁹ Toxins produced by *Fusarium* spp. were tested on *M. incognita* and some were highly toxic to

the nematode.⁵⁵ An antibiotic from *Cylindrocarpum olidum* (Wollenw.) was isolated, purified and characterized.^{70,517} It showed good nematocidal activity and had a low toxicity towards vertebrates.⁷⁰ A red pigment produced by *Verticillium suchlasporium* Gams & Dackman also has nematocidal properties.²⁸⁴

Endoglucases from *A. oligospora* were purified and characterized.²⁶⁵ Culture filtrates of *A. niger* and *Penicillium citrinum* Thom isolated from neem cake amended soil caused 100% mortality of juveniles of *Heterodera cajani* Koshy at standard concentration, and culture filtrates of *P. citrinum* also completely inhibited emergence of juveniles from cysts at all concentrations used.⁴³⁰ Purified extracts of a *Penicillium* sp., *Penicillium oxalicum* Currie & Thom, *Penicillium anatolicum* and *A. niger* showed high nematocidal activity at 100 and 200 ppm.³²¹ Although many of these fungi are parasites of nematodes, some are probably saprophytes rather than parasites. Presumably, many more soil saprophytic fungi that are antagonistic to nematodes through the release of toxins, antibiotics or enzymes remain to be discovered. Nematode density has been correlated negatively with activities of chitinase, collagenase, and proteinase of some soil microorganisms,^{312,393} including fungi such as *Cunninghamella elegans* (Mucorales).¹⁴⁹ Kloepper et al.²⁶⁰ observed that plants with properties antagonistic to plant-parasitic nematodes have rhizosphere microflora distinct from those of host plants; greater numbers of microorganisms in the rhizosphere of antagonistic plants were chitinolytic.²⁴¹ Whether the mycoflora are involved in the antagonism of the plant to nematodes through the release of substances is worthy of study.

Vesicular-arbuscular mycorrhizal fungi and other endophytes

The vesicular-arbuscular mycorrhizal (VAM) fungi are endomycorrhizal fungi that invade deeply into roots. The VAM fungi are all Zygomycetes, belonging to the order Glomales. The symbiotic association is obligate for these fungi, and they have not been successfully cultured apart from their hosts.³²⁴ During the last two decades many studies on their effects on nematodes have been reported. The roles of VAM fungi in regulating nematode populations and their modes of action have not been elucidated fully. The response of nematodes to VAM fungi varies and may depend on the specific association, soil nutrient level, and the timing of the observation.¹⁹⁴ Some reports suggested that VAM fungi have antagonistic effects on nematode populations.^{11,141,168,263,369,370,458} Population densities of *H. glycines* on soybean were unaffected or greater in microplots infested with VAM fungi than in untreated plots, whereas they were suppressed up to 73% in greenhouse tests.⁴⁸¹ *Meloidogyne incognita* numbers were reduced on cotton by a high inoculum (<60 spores/plant) of *Glomus fasciculatum* (Thaxter sensu Gerd.) Gerd. & Trappe⁴⁰⁹ in a greenhouse study. Carling et al.³⁹ reported that reproduction of *M. incognita* on soybean in a greenhouse was suppressed by VAM fungi at a low phosphorus rate. Jain and Sethi²⁰⁶ reported that the presence of *G. fasciculatum* had a profound adverse effect on cyst production and nematode multiplication of *H. cajani* on cowpea, whereas *Glomus epigaeum* Daniels & Trappe (= *Glomus versiforme* (Daniels & Trappe) Berch) tended to exhibit a reverse trend. Sitaranaiah and Sikora⁴³² that inoculation of tomato plants with *G. fasciculatum* significantly reduced juvenile penetration and development of *Rorylenchus reniformis* (Godfrey) Linford & Oliveira. Jain and Sethi²⁰⁷,

however, reported that the mycorrhizal fungi prevented nematode invasion, but had no effect on nematode development or fecundity. Other studies suggested that penetration of plant roots by nematodes was not affected by VAM fungi,^{435,459} but nematode development was retarded^{293,459} and nematode reproduction was lower in mycorrhizal than nonmycorrhizal roots.⁴³⁵ Jain and Sethi²⁰⁸ reported that early establishment of *G. fasciculatum* reduced gall formation by *M. incognita* and cyst formation of *H. cajani*, but prior establishment of *G. versiforme* resulted in increased numbers of cysts of *H. cajani*. The beneficial effects of VAM fungi were also reported on *Meloidogyne arenaria* (Neal) Chitwood^{9,229} and *M. incognita*.^{398,456} Other reports suggested that mycorrhizal infection had no effect on nematodes.^{46,186,491} Although VAM fungi were encountered in cysts and eggs, they do not appear to be important pathogens of nematodes.^{142,475} The VAM fungi may compete for nutrition¹⁹⁴ and root space, modify root exudates, alter plant physiology,^{11,194} colonize nematode feeding sites, reduce the number of giant cells,^{230,241,459} or release nematoxin⁴⁵⁹ or antibiotics.

Many fungi isolated from *H. glycines* cysts could colonize soybean roots.⁴⁴⁵ Some endophytic fungi, such as *Rhizoctonia solani* Kühn⁴⁹⁹ and *Rhizoctonia bataticola* (Taubenhaus) Butler,⁵⁰⁰ were also reported to have some adverse effect on nematode multiplication.

Fungal Species Isolated from Eggs, Egg Masses, Females, and Cysts

The existence of mycoflorae associated with eggs, egg masses, females, or cysts of nematodes belonging to the Heteroderidae has been known for more than a century.

As early as 1877, Kühn²⁶⁴ reported a fungus, *Tarichium auxiliarum* Kühn (now *Catenaria auxiliaris* (Kühn) Tribe), that parasitized females of *H. schachtii*.³³² Korab²⁶² first examined a total of more than 250,000 cysts of *H. schachtii* in 1929 and found that *Phialophora malorum* (Kidd & Beaumont) McColloch (as *Torula heteroderæ* sp. nov) was the principle fungal parasite. Goffart¹⁶³ reported *Cylindrocarpon destructans* (Zinssmeister) Scholten was the major fungus isolated from cysts of *H. avenae* in Germany in 1932. A total of 25,000 cysts of *H. schachtii* taken from Czechoslovakia were examined by Rozsypal⁴⁰² in 1934 and the major fungus was the same fungus found by Korab.²⁶² Recently, studies on the species and frequency of fungi associated with these nematodes have increased. Rodríguez-Kábana and Morgan-Jones³⁹⁴ reviewed the fungi isolated up to 1988 from Heteroderidae collected from Australia, Europe, and North and South America. Since then, many more fungal species have been reported from these nematodes collected in various geographic locations. The fungi most frequently isolated from eggs, females, egg masses and cysts of Heteroderidae are listed in Table 1-1. The fungal species most commonly isolated are limited to the following genera: *Acremonium*, *Alternaria*, *Catenaria*, *Cylindrocarpon*, *Exophiala*, *Fusarium*, *Gliocladium*, *Nematophthora*, *Paecilomyces*, *Penicillium*, *Phoma*, *Verticillium*.

The fungal species colonizing cysts of *H. glycines* have been isolated from the United States and China, two of the major soybean producing countries, and also from Colombia. At least 168 species of fungi have been isolated from *H. glycines* (Table 1-2). The common fungal species isolated from *H. glycines* are similar to those isolated from other cyst nematodes, except species of *Neocosmospora* and *Stagonospora*.⁵² Some

TABLE 1-1. Fungal species encountered at a relatively high frequency in eggs, females, egg masses or cysts of Heteroderidae.

Fungi	Nematode	Location	Citation
<i>Acremonium strictum</i>	<i>Heterodera schachtii</i>	USA	354
<i>Aspergillus flavus</i>	<i>H. schachtii</i>	Jordan	408
<i>Aureobasidium pullulans</i>	<i>Meloidogyne incognita</i>	USA	339
<i>Catenaria auxiliaris</i>	<i>H. schachtii</i>	Germany	466
		Europe	474
<i>Chaetomium cochliodes</i>	<i>H. glycines</i>	USA	158
<i>Coniothyrium fuckelii</i>	<i>Globodera rostochiensis</i>	Canada	56
<i>Cylindrocarpon destructans</i>	<i>G. rostochiensis</i>	Peru	333
(<i>Nectria ardicicola</i>)		England	76
	<i>G. pallida</i>	Peru	333
		England	76
	<i>H. glycines</i>	China	278
	<i>H. schachtii</i>	Poland	14
		England	33,75
		The Netherlands	187
		Spain	282
	<i>H. trifolii</i>	New Zealand	184
	<i>H. mediterranea</i>	Italy	498
(as <i>Cylindrocarpon radiculicola</i>)	<i>H. schachtii</i>	Germany	163
<i>Cylindrocarpon gracile</i>	<i>G. pallida</i>	Peru	333
	<i>G. rostochiensis</i>	Peru	333
	<i>H. glycines</i>	China	278
<i>Dactylella oviparasitica</i>	<i>Meloidogyne</i> spp.	USA	450,453
<i>Dictyochoeta coffeae</i>	<i>H. glycines</i>	USA	52
<i>Dictyochoeta heteroderæ</i>	<i>H. glycines</i>	USA	52
<i>Exophiala pisciphila</i>	<i>H. glycines</i>	USA	45,52,157,158,328
	<i>H. trifolii</i>	New Zealand	184
<i>Fusarium culmorum</i>	<i>H. trifolii</i>	New Zealand	184
<i>Fusarium oxysporum</i>	<i>G. rostochiensis</i>	Canada	56
		Czechoslovakia	367
	<i>H. glycines</i>	USA	45,157,158,326,328
	<i>H. schachtii</i>	Poland	14
		Jordan	408
		Spain	282
		USA	52,354
	<i>H. trifolii</i>	New Zealand	184
	<i>M. incognita</i>	USA	339
<i>Fusarium solani</i>	<i>G. rostochiensis</i>	Czechoslovakia	367
	<i>H. glycines</i>	USA	45,52,157,158,326,328
		China	278
	<i>H. schachtii</i>	Spain	282
		Jordan	408
	<i>H. trifolii</i>	New Zealand	184
	<i>M. incognita</i>	India	6
		Poland	14
<i>Gliocladium catenulatum</i>	<i>H. glycines</i>	USA	52
<i>Gliocladium roseum</i>	<i>G. rostochiensis</i>	Peru	333
		Czechoslovakia	367
	<i>G. pallida</i>	Peru	333
	<i>H. glycines</i>	USA	45,326

TABLE 1-1. (continued).

Fungi	Nematode	Location	Literature
<i>Gliocladium roseum</i>	<i>H. trifolii</i>	New Zealand	184
<i>Humicola grisea</i>	<i>G. rostochiensis</i>	Canada	56
<i>Mortierella alpina</i>	<i>H. trifolii</i>	New Zealand	184
<i>Nematophthora gynophila</i>	<i>H. avenae</i>	Sweden	87,90,242
		England	232,243,246
<i>Neocosmospora vasinfecta</i>	<i>H. glycines</i>	USA	52,157,326,328
<i>Olpidium vermicola</i>	<i>Nematodes</i>	Canada	24
<i>Paecilomyces lilacinus</i>	<i>H. glycines</i>	China	278
	<i>H. trifolii</i>	New Zealand	184
	<i>M. arenaria</i>	USA	161
	<i>M. incognita</i>	USA	339
	<i>Meloidogyne</i> spp.	USA	53
		Australia	309,454
<i>Paraphoma radicina</i>	<i>H. glycines</i>	USA	45
<i>Penicillium verrucosum</i> (as <i>Phoma leveillei</i>)	<i>G. rostochiensis</i>	Czechoslovakia	367
		USA	157
<i>Phialophora malorum</i> (as <i>Torula heteroderae</i>) (as <i>Trichosporium populi</i>)	<i>H. schachtii</i>	USSR	262
		Czechoslovakia	402
<i>Phytophthora cinnamomi</i>	<i>H. glycines</i>	USA	158
<i>Pyrenochaeta terrestris</i> (As <i>Phoma terrestris</i>)	<i>H. glycines</i>	USA	45,52
		USA	158
<i>Septocylindrium-like fungus</i>	<i>G. rostochiensis</i>	Sweden	88
<i>Stagonospora heteroderae</i>	<i>H. glycines</i>	USA	45,52,326
		China	278
<i>Trichocladium opacum</i>	<i>H. trifolii</i>	New Zealand	184
<i>Trichoderma</i> sp.	<i>Meloidogyne</i> sp.	Korea	259
<i>Trichosporon beigeli</i>	<i>H. glycines</i>	USA	158
<i>Ulocladium atrum</i>	<i>G. pallida</i>	Peru	333
	<i>G. rostochiensis</i>	Peru	333
<i>Verticillium chlamydosporium</i>	<i>H. avenae</i>	Sweden	87,90,242
		Germany	421
		England	232,246
	<i>H. glycines</i>	USA	45
	<i>H. schachtii</i>	England	33
		The Netherlands	187
		Jordan	408
		Spain	282
		Germany	466
	<i>H. trifolii</i>	New Zealand	184
	<i>Meloidogyne</i> spp.	Australia	309
<i>Verticillium suchlasporium</i>	<i>H. avenae</i>	Sweden	87,242
<i>Verticillium</i> sp.	<i>H. avenae</i>	Sweden	90
A black yeast (close to <i>Phialophora malorum</i>)	<i>H. schachtii</i>	Germany	485
A black yeast-like fungus	<i>H. glycines</i>	USA	52
An unknown fungus (close to <i>Phialophora heteroderae</i>)	<i>G. pallida</i>	Germany	166
Arkansas Fungus 18	<i>H. glycines</i>	USA	256

TABLE 1-2. Fungal species isolated from the soybean cyst nematode, *Heterodera glycines*.

Genus	Species	literature cited
<i>Acremonium</i>	<i>kiliense</i>	45,280
	<i>sclerotigenum</i>	45
	<i>strictum</i>	158,280
<i>Alternaria</i>	<i>alternata</i>	45,158,328
	sp.	278
<i>Aphanocladium</i>	<i>album</i>	45
<i>Arthrobotrys</i>	<i>oligospora</i>	45
<i>Arthrocladium</i>	<i>caudatum</i>	45
<i>Aspergillus</i>	<i>flavus</i>	158
	<i>fumigatus</i>	334
	<i>niger</i>	334
	spp.	278
<i>Aureobasidium</i>	<i>pullulans</i>	45,278
<i>Botryotrichum</i>	<i>piluliferum</i>	45
<i>Camposporium</i>	<i>pellucidum</i>	45
<i>Catenaria</i>	<i>auxiliaris</i>	83,278
<i>Chaetomium</i>	<i>cochliodes</i>	45,52,158
	<i>globosum</i>	157
	<i>gracile</i>	326
	<i>histoplasmaeoides</i>	45
	<i>indicum</i>	160
	<i>perlucidum</i>	45
	sp.	311
<i>Chalara</i>	<i>heteroderae</i>	45
	<i>hyalina</i>	158
<i>Cladosporium</i>	<i>cladosporioides</i>	45,326
	<i>sphaerosporium</i>	158
<i>Corynespora</i>	<i>casticola</i>	45,278
<i>Cristaspora</i>	<i>arxii</i>	45
<i>Curvularia</i>	<i>lunata</i>	52,326,334
<i>Cylindrocarpum</i>	<i>destructans</i>	45,273,278
	<i>didymum</i>	278
	<i>fusiforme</i>	45
	<i>gracile</i>	278
	<i>heteronema</i>	278
	<i>magnusianum</i>	43,45
	<i>tonkinense</i>	158
	<i>wilkinsonii</i>	278
	sp.	278
<i>Dactylaria</i>	<i>acerosa</i>	45
<i>Dendryphon</i>	<i>nanum</i>	45
<i>Dictyochoeta</i>	<i>coffae</i>	52
	<i>heteroderae</i>	45,52
	(= <i>Cordinia heteroderae</i>)	160,326
<i>Drechslera</i>	<i>avenae</i>	45
	<i>fugax</i>	52
<i>Engyodontium</i>	<i>album</i>	45
<i>Epicoccum</i>	<i>purpurascens</i>	45
	sp.	311
<i>Exophiala</i>	<i>jeanselmei</i>	311
	<i>pisciphila</i>	45,52,157,158,326,328

TABLE 1-2. (continued).

Genus	Species	literature cited
<i>Fusariella</i>	<i>bizzozzerizna</i>	278
<i>Fusarium</i>	<i>aquaeductum</i>	45
	<i>equiseti</i>	45,52,157,278,326,334
	<i>lateriterum</i>	157,278,326,334
	<i>moniliforme</i>	334
	<i>oxysporum</i>	45,52,157,158,160,278,326,328,334
	<i>semitectum</i>	52
	<i>solani</i>	45,52,157,158,278,326,328,334
	<i>udum</i>	278
	spp.	52,311
	sp.	45
<i>Geniculosporium</i>		45
<i>Geotrichum</i>	<i>candidum</i>	158,334
<i>Gliocladium</i>	<i>catenulatum</i>	45,52,160,326,334
	<i>roseum</i>	45,158,160,326,334
<i>Glomus</i>	<i>fasciculatum</i>	142
	sp.	278
<i>Gonytrichum</i>	<i>macrocladium</i>	45,52
<i>Helicomycetes</i>	sp.	52
<i>Humicola</i>	<i>fuscoatra</i>	45,158
	<i>grisea</i>	157
	spp.	52,278
<i>Lecythophora</i>	<i>hoffmannii</i>	45
<i>Macrophomina</i>	<i>phaseoli</i>	326
	<i>phaseolina</i>	158
<i>Mariannaea</i>	<i>elegans</i>	45,158
<i>Melanospora</i>	<i>zamia</i>	45,158
<i>Memnoniella</i>	<i>echinata</i>	334
<i>Metarhizium</i>	<i>anisopliae</i>	45
<i>Microsphaeropsis</i>	<i>olivacea</i>	45,51,158
<i>Monacrosporium</i>	<i>bembicoides</i>	158
<i>Mortierella</i>	<i>elongate</i>	45
<i>Mucor</i>	sp.	158
<i>Mycocentrospora</i>	<i>acerina</i>	45,158
<i>Myrothecium</i>	<i>verrucaria</i>	158,326
	sp.	52
<i>Nectria</i>	sp.	43
<i>Nematophthora</i>	<i>gynophylla</i>	83
<i>Nematophthora</i>	sp.	278
<i>Neocosmospora</i>	<i>vastifecta</i>	43,52,157,158,160,273,326,328
<i>Nigrospora</i>	<i>spharica</i>	45
<i>Paecilomyces</i>	<i>inflatus</i>	51
	<i>lilacinus</i>	45,52,158,278,334
	<i>marquandii</i>	45
	<i>variotii</i>	158,280
<i>Papulaspora</i>	spp.	45,52
<i>Paraphoma</i>	<i>radicina</i>	45,52,158
<i>Paraphoma</i>	sp.	52
<i>Passerinula</i>	sp.	45
<i>Penicillium</i>	<i>aurantiogriseum</i>	158
	<i>decumbens</i>	158
	<i>oxalicum</i>	45

TABLE 1-2. (continued).

Genus	Species	literature cited
<i>Penicillium</i>	<i>restrictum</i>	158
	<i>simplicissimum</i>	158
	<i>steckii</i>	328
	<i>verrucosum</i>	334
	spp.	51,52,278
<i>Periconia</i>	<i>macrospinosia</i>	45,52,157
<i>Pestalotiopsis</i>	sp.	158
<i>Phaeoisaria</i>	<i>clematidis</i>	45
<i>Pharoanularia</i>	sp.	45
<i>Phialophora</i>	<i>gregata</i>	45
<i>Phoma</i>	<i>americana</i>	158
	(=Black yeast I)	326
	<i>chrysanthemicola</i>	52,311
	<i>eupyrena</i>	157,278
	<i>leveillei</i>	157,278
	<i>macrostoma</i>	157,160,326
	<i>medacaginis</i>	43,157,334
	<i>multirostrata</i>	160,326
	<i>pinodella</i>	45
	<i>pomorum</i>	157
	spp.	52,278
<i>Plectosphaerella</i>	<i>cucumerina</i>	45
<i>Phyllophora</i>	sp.	278
<i>Phytophthora</i>	<i>cinnamomi</i>	158
<i>Pithomyces</i>	sp.	278
<i>Pseudurotium</i>	<i>ovale</i>	158
<i>Pseudorobillarda</i>	<i>sojae</i>	52
<i>Pyrenochaeta</i>	<i>terrestris</i>	45,52
	(=Phoma terrestris)	158
<i>Pythium</i>	sp.	158
<i>Ramichloridium</i>	<i>schulzeri</i>	45
	<i>subulatum</i>	52
<i>Rhizoctonia</i>	<i>solani</i>	52,326
	sp.	278
<i>Sagenomella</i>	<i>hanlinii</i>	158
	<i>levispora</i>	334
<i>Scolecobasidium</i>	<i>terreum</i>	51,157
	<i>tricladiatum</i>	51
<i>Scopulariopsis</i>	<i>brevicaulis</i>	334
<i>Scytalidium</i>	<i>fulvum</i>	158,311
	<i>lingicola</i>	52
<i>Septonema</i>	<i>chaetospora</i>	45
<i>Sistotrema</i>	<i>brinkmannii</i>	45
<i>Stachybotrys</i>	<i>elegans</i>	278
<i>Stagonospora</i>	<i>heteroderae</i>	45,52,160,278,326,334
<i>Staphylotrichum</i>	<i>cocosporum</i>	45
<i>Stilbella</i>	<i>bulbicola</i>	45
<i>Thielavia</i>	<i>basicola</i>	326
	<i>ovispora</i>	45
	<i>terricola</i>	157,160,326
<i>Trematosphaeria</i>	<i>fallax</i>	45

TABLE 1-2. (continued).

Genus	Species	literature cited
<i>Tricellula</i>	<i>inaequalis</i>	43
<i>Trichocladium</i>	<i>asperum</i>	334
	<i>opacum</i>	45,278
<i>Trichoderma</i>	<i>harzianum</i>	158,326,328
	<i>koningii</i>	45
	<i>lignorum</i>	52
<i>Trichosporon</i>	<i>beigleii</i>	158
<i>Tritirachium</i>	<i>oryzae</i>	45
<i>Verticillium</i>	<i>chlamydosporium</i>	83,158,278
	(= <i>Diheterospora chlamydosporia</i>)	45
	<i>lamellicola</i>	160,326
	<i>lecanii</i>	158
	<i>leptobactrum</i>	160,328
<i>Volutella</i>	<i>ciliata</i>	45,278
Black yeast (II)		326
Sterile fungus 1 (a black yeast-like fungus)		52
Sterile fungus 2 (an extremely slow-growing fungus)		52
Sterile fungus 3 (Arkansas Fungus 18)		256

studies indicated that mycoflora in young females of *H. glycines* are different from that in older females or cysts.^{52,157,158} As the females become more exposed to the soil, they were increasingly vulnerable to fungal infection.

Modes of Infection

The knowledge of mode of infection of nematodes by fungi is important for using them as biological control agents. Nordbring-Hertz and her colleagues^{30,216,217,219,220,358-365,381,472,478-480,486,488-490} did extensive studies on the mechanism of fungal infection of nematodes, especially on trapping and endoparasitic fungi attacking vermiform nematodes. The relationships of *A. oligospora* or *Meria coniospora* Drechsler (= *Drechmeria coniospora* (Drechsler) Gams & Jansson) with nematodes were used as models for studying the recognition mechanism of fungal infection.^{360,362}

Attraction. Attraction between nematodes and nematophagous fungi was observed in many studies.^{18,138,211,212,215,216,218,226,519,526} The attraction of fungi to nematodes was correlated with the ability of nematophagous fungi to destroy nematodes.²¹¹ The attraction intensity increased with increasing dependence of the fungi on nematodes for nutrients.^{215,216} Several factors, such as carbon dioxide and various organic and inorganic substances, have been thought to be involved in nematode attraction.^{18,176} A volatile or a small rapidly diffusing compound was continuously produced in nonspontaneous trap formers (fungi insensitive in trap formation in response to organic matter, including net-forming species¹⁷⁵). Spontaneous trap formers (fungi sensitive in trap formation in response to organic matter, including branch-, knob- and ring-forming species¹⁷⁵) and

endoparasites produced larger, or less volatile, slowly diffusing compounds, which attract nematodes.^{176,215} Sialic acid located on the head region of nematodes was found to be involved in chemotaxis of zoospores to nematodes.²¹⁹ The motile zoospores of endoparasitic fungi are thought to be attracted to nematodes as a result of the chemical gradients formed by exudates released from nematode body orifices.⁴⁴⁸ It is not clear if there is a chemotropic response of facultative fungal parasites of eggs or females towards their sedentary hosts.³⁶⁰ Zoospores of *C. auxiliaris*⁴⁷⁴ and *N. gynophila*²³¹ are thought to move toward nematode females.

Attachment. A review on fungal attachment to nematodes was presented by Tunlid et al.⁴⁷⁸ When nematodes come in contact with nematophagous fungi, some morphological changes and chemical interactions may occur. The binding sites vary among combinations between nematodes and fungi.¹²⁹ Three patterns of binding sites of nematodes with fungi have been observed: 1) specifically to the head and tail, 2) all over the body, and 3) very sparse or no binding.²¹³ Zoospores usually attach near natural body openings.^{105,448} Molecular moieties are responsible for recognition between nematodes and nematophagous fungi.^{213,219,478,526} Although it had been suggested previously that nematode-trapping fungi were not nematode specific,⁴⁰⁰ the results obtained by Gaspard and Mankau¹⁵⁴ indicated that host-specificity does exist in the interaction between trapping fungi and nematodes. Binding of lectin-carbohydrates is involved in recognition between trap fungi and nematodes.^{129,362,503} Lectin has been purified and characterized from fungal trap cells.³⁹⁹ The qualitative and quantitative variation in carbohydrates on the surface of different nematodes may provide more

opportunity for ecological specialization of endoparasitic fungi that use lectins in host recognition.¹²⁹ Attachment and penetration of nematodes by fungi are two separate events.²¹³ Blocking the binding sites by saccharides prevented penetration of *Trichostrongylus colubriformis* (Giles) Ransom by *A. oligospora*, but had no effect on capture of the nematode; thus capture and penetration may be two distinct processes, with capture being less specific than penetration.⁵⁰³ Freeze-dried conidia of *D. coniospora* attached to *Caenorhabditis elegans* (Maupas) Dougherty and *Panagrellus redivivus* (L.) Goodey as readily as did untreated conidia, but infection was significantly delayed.⁵²⁴

Penetration. Many light and electron microscopic studies have been published showing the penetration of nematode cuticle by different nematophagous fungi.²²⁰ Whether penetration of the cuticle is enzymatic or mechanical, or both, is not yet clear.²²⁰ Organelles such as endoplasmic reticulum in infective hyphae proliferated at a high rate during the infection process, which suggests the involvement of enzymatic activities.^{486,487,490} But some studies have shown that these organelles are not involved in either the secretion of adhesives or the lysis of the cuticle.²²⁰ Protein changes of *C. elegans* and *D. coniospora* were observed during infection.⁵⁹ Some observations suggested that mechanical force is involved in the penetration of the nematode cuticle.^{220,345,488,489} *Hirsutiella rhossiliensis* spores are adhesive to vermiform nematode cuticle before they penetrate the cuticle. A bulbous infection hypha formed from the spore, and secondary hyphae developed from the hypha.²⁰⁴

Females and cysts may be colonized by one of the following ways: (1) directly through the cuticle; (2) by penetration through the natural body openings of the vulval

cone or oral opening; and (3) after colonization of the feeding cell within the root, which may itself cause the death of the female.²³⁷ Only a few fungi have been shown to penetrate the cuticular wall of cysts.^{183,258}

Enzymes and mechanical activities may be involved in the penetration of eggs, although the processes are poorly understood. Chitinolytic activity is probably important in dissolving the egg shell.^{160,266,330} Fungi without any chitinolytic activity probably penetrate only the mechanically damaged eggs.²⁶⁶ Protease may be involved in nematode egg penetration by *V. suchlasporium*.^{283,289} Kunert and Lysek²⁶⁷ showed no evidence for the involvement of lipolytic enzymes in the penetration of the nematode egg shell by parasitic fungi. Appressoria formed by *V. suchlasporium* and *V. chlamydosporium* were involved in the penetration of nematode eggs.^{286,287,289-291} Lysek and Sterba²⁹² demonstrated that no penetration organs were involved in the penetration of an egg shell of *Ascaris lumbricoides* L. by fungi. Appressoria were also formed on nematodes by *Dactylella oviparasitica* Stirling & Mankau when the fungus contacted nematode eggs, and mechanical activity was involved during the penetration.⁴⁵² Morgan-Jones et al.³³⁹ observed that *P. lilacinus* penetrated *M. arenaria* eggs through small pores dissolved in the vitelline layer. Dunn et al.¹²⁷ showed that *P. lilacinus* colonized *M. incognita* eggs through the formation of appressoria or simple hyphal penetration. Zoospores of *Catenaria anguillulae* Sorokin encysted on nematode eggs by chance and germinated in such a way that the germ tube penetrated the lipid layer; other zoospores were attracted to chemotactic substances leaking out. The embryo was then killed within a few minutes following mass aggregation and encystment of the zoospores.⁵¹⁵ Eggs parasitized by

nematophagous fungi may become filled with mycelium, discolored, or misshaped.⁴⁰⁷

Pathogenicity of fungi to nematodes

Once a fungus penetrates a nematode, the fungus usually proliferates within the nematode and then kills and consumes the nematode body contents. The pathogenicity varies among the various nematophagous fungi. Among the trapping fungi those that form unmodified adhesive hyphae or branches are considered primitive types, whereas fungi forming constricting rings are more evolved and very aggressive.¹⁷⁶ Ingestion of nematodes by predaceous fungi is often completed within 48 to 72 hours.²²⁰ Most endoparasites of vermiform nematodes are obligate parasites, and they are dependent on nematodes for nutrition.

Only a few studies have been reported on biochemical processes of assimilation of nematode body contents by fungi. Hydrolytic enzymes are not likely to have a function in the digestion of nematode body contents.⁴⁸⁹ Oxidation of lipids, however, occurs later or during development of new vegetative mycelium.²²⁰

Some fungi isolated from cysts and eggs of Heteroderidae are saprophytic or weak pathogens. Many, however, are capable of parasitizing sedentary nematode females and eggs.^{33,87,88,136,143,144,158,160,161,195,243,246,248,256,311,327,329,337-339,354,383,396,412,449,454,496,502,513}

Species of *Verticillium* can parasitize and destroy eggs and juveniles of many nematodes.^{33,87,88,143,158,161,246,291,292,310,311,327,329,337,383,449,454} *Cylindrocarpon destructans*

penetrates and destroys eggs of *H. schachtii*^{33,243} and *Meloidogyne javanica* (Treub) Chitwood.¹⁴⁴ The pathogenicity of *P. lilacinus* has been tested on various nematodes.^{36,}

143,144,329,412,454

Variations in virulence were observed among strains of *V.*

chlamydosporium,^{195,248} *P. lilacinus*,^{36,94,144,396,412,454,502} *C. destructans*,³⁹⁴ *Fusarium oxysporum* Schlecht.,^{160,354,412} *Fusarium solani* (Mart.) Sacc.,¹⁶⁰ *Gliocladium roseum* Bain,³⁹⁶ *C. anguillulae*,⁴⁹⁶ and *Acremonium strictum* Gams.³⁵⁴ Variation in virulence among isolates of a fungal species against nematodes appears to be the rule rather than the exception.³⁹⁴

Frequently, subculturing on artificial medium may reduce virulence of *C. anguillulae* to nematodes.⁴⁹⁷ Similar results were observed with *D. coniospora*.⁵²⁵ The maintenance of virulence in nematophagous fungi can best be assured by cryopreservation of subcultures soon after isolation from the natural environment.⁵²⁵

Ecology of Nematophagous Fungi

The nematophagous fungi are worldwide in distribution and have been reported from many countries, including the United States,^{45,52,113,114,135,157,158,161,201,259,301,322,323,326,328,339,451,471} Canada,^{19,24,56,134,468} Brazil,^{346,413,429} Argentina,¹⁵¹ Peru,^{222,333,483} Colombia,³³⁴ England,^{116-121,228,231,244} Scotland,^{28,285,294} Ireland,^{169,170,172} Italy,^{391,492} Poland,^{14,221} Germany,^{10,128,145,146,163,166,264,421,457,466,485} France,^{60,376-378} former USSR,^{181,262,306,307,436,437} Denmark,⁴²⁷ Finland,^{403,410} Sweden,^{87,242} Spain,²⁸² The Netherlands,¹⁸⁷ Czechoslovakia,^{402,495} India,^{6,93,95-98,319,404,405,415,440} Malaysia,²⁶⁹ Japan,^{261,317,318,320} China,^{185,273,278,280,502,518,523} Korea,²⁵⁹ Pakistan,^{344,522} Jordan,⁴⁰⁸ the Philippines,⁴⁹⁴ Australia,^{304,449,454,455,461} New Zealand,^{21,140,184,510} and Maritime Antarctic.^{178,179}

Nematophagous fungi have been recovered from various habitats. Predaceous fungi were isolated often from leaf mold, decaying wood, partly decayed plants, dung,

and living bryophytes, and soil.¹²⁰ Gray and Bailey¹⁷⁷ surveyed the distribution of nematophagous fungi in a deciduous woodland. They found that nematophagous fungi were isolated throughout the soil profile and down to a maximum of 35 cm deep. Predators forming constricting rings, adhesive branches and adhesive knobs are restricted to the upper litter and humus layers. The net forming predators and endoparasites are isolated throughout the soil profile at all depths, although they were more abundant in the deeper mineral rich soil.

Cooke^{62,63} hypothesized that competition stimulates the predatory activity of nematode-trapping fungi as an adaptation to overcome their low competitive saprophytic ability. This hypothesis was supported by some observations.^{64,384} It appears that the development of predaceous efficiency has been accompanied by the tendency to lose characters such as rapid growth rate and good competitive saprophytic ability that are associated with an efficient saprophytic existence in the soil.⁶⁴ The ability of nematophagous fungi to destroy nematodes was correlated with attraction of the fungi to nematodes.²¹¹ The attraction intensity increased with increasing dependence of the fungi on nematodes for nutrients.^{215,216} Belder and Jansen²⁶ observed that formation of trapping devices was less temperature- and nutrient-dependent in simple adhesive hyphae than in complex adhesive network.

Barron²² argued that many nematophagous fungi evolved from lignolytic and cellulolytic fungi. The evolutionary process is a response of these fungi to nutrient divergence in N-limiting habitats. This argument was based mainly on a series of observations by Cooke.^{62-65,414} Although the teleomorphs of most predaceous

Hyphomycetes are not known, many predaceous fungi are members of the gilled fungi.^{405,468} The teleomorphs of *Nematoctonus* spp. are gilled fungi, *Hohenbuehelia* spp.²³ Many fungi occurring in rotting wood prey on nematodes.^{415,468} Barron and Thorn²⁵ observed that the adhesive knob-forming fungus, *Pleurotus ostreatus* Jacquin ex Fries (oyster mushroom), has developed a unique method of inactivating and eventually colonizing its nematode victims. Nematodes were paralyzed by a toxin release from minute spatulate secretory cells of the fungus. Thorn and Barron⁴⁶⁸ reported from analyses of herbarium specimens that species of gilled fungi *Resupinatus* and *Stignatolemma* also possessed secretory-like cells on their hyphae that closely resembled those of *P. ostreatus*. It is probable that the method of capturing nematodes developed by *P. ostreatus* is more common than currently known.²² All of these fungi are lignolytic.

Nematophagous fungi are affected by many soil variables, such as soil moisture, organic matter, pH, nematode density, soil nutrients, metals, and temperature,^{175,241} and by plant host,³⁹⁴ cropping history,^{394,444} and climatic conditions.³⁹⁴ Crump and Kerry⁸¹ tested the effects of 25 chemicals on the growth of *V. chlamydosporium*. Of the nematicides tested, oxamyl was the only one that decreased growth of *C. destructans* and *V. chlamydosporium*. The insecticides and herbicides tested had little effect on the fungal growth. The densities of obligate parasites depend on the density of their hosts. Some fungi with weak saprophytic habits, such as *H. rhossiliensis*, and probably some trapping fungi are partially density-dependant.¹⁹⁸ The occurrence of facultative fungi, such as those isolated from eggs, sedentary females, and cysts, are mainly influenced by

environmental factors,⁵² and are independent of nematode density.³⁵² However, relatively few genera have been associated with more than one species of nematodes,³⁹⁴ and the common fungal species isolated from cysts of the *H. glycines* collected from different geographic locations are similar.⁵² It is suggested that a degree of specialization may be a prerequisite for successful colonization of the ecological niche represented by eggs, females, or cysts of heteroderid nematodes.³⁹⁴

Additional information about the ecology of nematophagous fungi is provided by Duddington,¹²⁰ Gray,^{175,176} Nordbring-Hertz,³⁶⁰ Jaffee et al.,^{196,203} Barron,^{18,22} Cooke,^{63,64} and Kerry.²⁴⁰

Biological Control of Nematodes by Fungal Antagonists

Since Linford's^{274,277} demonstrations that nematode populations were reduced by addition of organic matter to soil, which enhances the activity of nematophagous fungi, much effort has been made to search for biological control antagonists, to introduce nematophagous fungi into soil, and to enhance activities of nematophagous fungi by the addition of some soil amendments. Much of the early research was conducted on fungal predators and endoparasites of vermiform nematodes. Although a few were successful^{5, 154,214,473} or partially successful,^{122,224,275,277} the majority were unsuccessful.^{123-126, 182,192,276,296,349,463} The failures probably occurred because the predator and endoparasites of vermiform nematodes were poor competitors in the soil and a simple labile equilibrium between nematode density and trapping activity did not exist.¹⁷⁵ More studies on the ecology and diversity of species are needed before reaching conclusions of

whether these fungi can be used for biological control of nematodes. Some successful natural and induced biological control agents have been identified, but none has been accepted widely. Several reviews and book chapters are available on biological control of nematodes by fungal antagonists.^{175,233-236,240,241,299,331,447,448,477}

Natural control of nematodes by fungi

Nematodes and their fungal antagonists in soil have coevolved for a long time. The equilibrium between nematode density and some nematophagous fungi may exist in undisturbed soil or in soil with continuing monoculture. Although a few surveys have demonstrated the prevalence of natural biological control agents, the mechanisms resulting in nematode suppressive soils are poorly understood.

The decline of the cereal cyst nematode *H. avenae* has been well documented in Europe.^{147,209,368} Four species of parasitic fungi, *N. gynophila*, *V. chlamydosporium*, *C. destructans*, and *C. auxiliaris*, were found to be responsible for the decline of cereal cyst nematode,^{232,243} but *N. gynophila* and *V. chlamydosporium* were the most important.^{246,448} Boag and Lopez-Llorca²⁸ reported that *Verticillium* sp., *N. gynophila*, *Paecilomyces carneus* (Duché & Heim) Brown & Smith, and *C. destructans* were responsible for controlling the population densities of *H. avenae* below economic threshold levels in eastern Scotland. Although sugar beet cyst nematodes were suppressed by fungal pathogens, mainly *C. destructans*, *V. chlamydosporium*, *N. gynophila*, and *C. auxiliaris*,⁸⁰ the soil containing these fungal pathogens did not cause *H. schachtii* population densities to fall below the damage threshold.⁸² The suppression of nematodes by fungi have also been reported from other nematodes and many countries.^{45,131,391,450,455}

Enhanced biological control

Linford²⁷⁴ demonstrated that the addition of organic matter to soil resulted in marked changes in the indigenous nematode populations. Soil amendments increased free-living nematodes and decreased plant-parasitic nematodes.¹⁷⁵ It was suggested that the organic supplement, which led to the increase in nematode density, also resulted in increased nematophagous fungal activity in the soil. The enhanced activity of the nematophagous species present in the soil would then stabilize the rate of increase of the soil nematodes, and eventually reduce their population densities, including those of plant-parasitic nematodes.¹⁷⁵ This hypothesis has been used as the basis for a number of experiments on the biological control of plant-parasitic nematodes in soil. Many early experiments on biocontrol of plant-parasitic nematodes by addition of soil organic materials failed.^{123-126,182,192,276,296,463} Cooke's^{61-63,66} series of experiments suggested that in nature a simple labile equilibrium does not exist. Recent work indicates that equilibria do exist between nematophagous fungi and soil nematodes.^{175,196,197} Some studies inferred that dependency on nematodes density^{64,148,211,215} and response to soil organic amendments^{64,175} vary among the trapping fungi. Species that forms either a branch, knob, or constricting ring is sensitive in forming traps in soil amended with organic matter, whereas net-forming fungi are not.¹⁷⁵ The mode of action of organic amendments against nematodes consists of more than the direct effects on nematophagous fungi. Organic amendments can improve soil structure and soil fertility, alter the level of plant resistance, release toxic compounds, and stimulate antagonistic micro-organisms.⁴⁴⁸ All of these changes by organic amendments may affect nematodes dramatically.

Recently, studies of the effects of soil amendments on egg or female parasites have increased. When chitin was added into soil, population densities of *M. incognita*,³⁰² *M. arenaria*,^{85,86,162,312,393} *M. javanica*,¹⁴⁹ *H. glycines*,³⁹⁵ *H. avenae*,⁴³⁹ *Tylenchulus semipenetrans* Cobb,⁴³⁹ *Tylenchorhynchus dubius* (Butschli) Filipjev,³¹⁴ *Pratylenchus brachyurus* (Godfrey) Filipjev & Schuurmans Stekhoven,³⁹³ and *Pratylenchus penetrans* Cobb³¹⁴ were suppressed. The root-galling index of tomato inoculated with *M. javanica* was decreased 70 % when collagen was used as a soil amendment and 90 % when the amendment was supplemented with the collagenolytic fungus, *Cunninghamella elegans*.¹⁴⁹ Although the mechanisms of the suppression of nematodes by these soil amendments are not clear, chitinolytic or collagenolytic and other enzymatic activities of soil organisms, including fungi, increased after addition of the amendments,^{149,162,312,438} and the changes of enzymatic activities of soil microorganism are thought to be responsible for the suppression of nematode populations.^{312,393} Godoy et al.¹⁶² observed that the elements of stimulated mycoflora previously associated with parasitism of eggs of *H. glycines* and *M. arenaria* were isolated from chitin-amended soil. But the exact relationship between nematodes and the micro-organisms stimulated by chitin remained obscure.⁴⁴⁸ Toxin produced by addition of organic soil amendments may be another factor effecting the nematode population.³⁰² Reduction of population densities of root-knot and cyst nematodes by addition of animal manures, green manure, and oil cake was also obtained.^{5,372,431,433} Some studies disproved the idea that organic amendments stimulated the fungal parasitism of eggs or females.^{296,353,395}

Introduction of fungal biological control agents

Numerous fungi, including various types of fungal antagonists of nematodes, have been tested for their efficacy for control of plant-parasitic nematodes,^{1,3-5,37,72,76,77,86,94,100,101,103,107,115,180,182,189,193,223,252,254,256,373,387,396,397,423,425,441,462,494,497,514,521} but none of them has been accepted widely. Nematologists continue to debate whether manipulation of natural enemies in soil is likely to be more successful than the addition of selected agents used to inundate pests.²⁴¹ Kerry²⁴¹ believed that, at this stage in our knowledge, both approaches appear valid and much can be learned from either.

Fungi usually have been introduced into soil with some type of nutritional carriers. They can also be introduced into soil with seeds.⁶⁹ Molasses or brewer's yeast were used as culture media for mass production of fungal biological control agents against soil-borne pathogens.³⁷⁴ Nematophagous fungi can be formulated in alginate granules.^{271,422,423} A formulated biological control agent was tested in the field and was reported to reduce nematode population densities.⁴ Commercial products, Royal 300 containing *A. superba* and Royal 350 containing *A. irregularis*, were developed for control of root-knot nematodes^{47,375} on vegetables and *Ditylenchus myceliophagus* Goodey on mushrooms.⁴⁹

Paecilomyces lilacinus has been tested widely for its potential as a biological control agent. A formulation called Biocon, containing *P. lilacinus*, is being sold for control of root-knot and cyst nematodes in the Philippines.⁴⁶⁹ The fungus was first observed in association with nematode eggs by Lysek⁴⁰⁰ and is a species with cosmopolitan distribution, particularly in the warmer regions of the world.⁴⁴⁸

Paecilomyces lilacinus appears to be a good root colonizer³⁵ and rhizosphere competitor, however, its depth of distribution in sandy soils appears to be limited to the upper 15 cm.¹⁸⁹ The fungus has been shown to suppress population densities of *Meloidogyne* spp.,^{37,72,115,193,223,254,373,397,425,443,462,494,520,521} *Globodera rostochiensis* (Wollenweber) Behrens,^{94,180,252} *R. reniformis*³⁸⁷ and increase plant yield in microplots.³⁷ However, not all tests with *P. lilacinus* have been successful.^{107,189} The variation of experimental results may be attributed to the variation of virulence among isolates.^{36,94,454} A strain of *P. lilacinus* was shown to suppress *M. incognita* and *M. arenaria* populations but not *M. javanica*.⁵¹⁴ The number of fungal propagules in the soil was correlated to the initial dose applied and decreased progressively through time with increased dose.¹⁶⁵ Culbreath et al.⁸⁶ demonstrated that effective control of *M. arenaria* was not obtained by *P. lilacinus* alone, but the nematode was controlled by combination of the fungus with chitin as soil amendment. *Meloidogyne incognita* was reported to be controlled effectively when used in combination with *Pasteuria penetrans* (Thorne) Sayre & Starr.¹¹⁵

Optimal temperature for *P. lilacinus* growth and sporulation is 25-30 C, and the satisfactory pH range is 5-9.⁵⁰¹ Villanueva and Davide⁴⁹³ reported that optimum mycelial growth occurred at pH 6-9 and optimum sporulation occurred at pH 4-6. This fungus can grow well on various plant substrates, such as corn grits, rice hulls, chopped water lily, mashed potatoes and ipil-ipil leaves.⁴⁹⁴ The pathogenicity of *P. lilacinus* to human eyes and sinuses is a limitation for using this fungus as a biological control agent.^{2,313,340,347,392}

Verticillium chlamydosporium is an ubiquitous fungus and has been found on

various nematodes, including cyst,^{33,45,83,87,158,184,187,210,232,242,246,278,282,329,408,421,449,466,509} and root-knot^{161,309,327,329,383,391,454,522} nematodes in soil. The fungus is thought to be responsible for suppression of *H. avenae* in Europe,^{232,243} but it does not efficiently suppress the population of *H. schachtii*⁸² or *Meloidogyne* spp.^{153,155} in naturally infested soil. Since demonstration of pathogenicity of *V. chlamydosporium* to the cyst nematodes, *H. schachtii*⁵⁰⁵ and *H. avenae*,²³² many studies have been done to evaluate potential of this fungus as a biological control agent against nematodes. In greenhouse studies, introduction of *V. chlamydosporium* into soil gave significant population control of *H. avenae* on wheat,²⁵¹ *H. schachtii* on oilseed rape and beet,⁵⁷ *M. incognita* on tomato,^{100,104} and *M. arenaria* on tomato¹⁰³ and squash.¹⁶¹ The greatest control of *Meloidogyne* spp. was obtained at 25 C.¹⁰¹ Different isolates varied in their pathogenicity to the eggs of *H. avenae*.^{195,248} Although studies by Kerry and his colleagues^{103,159} suggested that *V. chlamydosporium* can not invade the root cortex and is confined to the rhizoplane, Lopez-Llorca and Boag²⁸⁵ reported that *V. chlamydosporium* invades roots of wheat, oat, and barley before infecting *H. avenae* females.

Hirsutella rhossiliensis has been found on *H. schachtii* in Germany³⁴³ and the United States,^{200,201} on *Xiphinema diversicaudatum* (Micoletzky) Thorne in Italy,⁵⁴ on *H. avenae* in Australia,⁴⁴⁹ and on *Criconebella xenoplax* Raski in the United States.^{131,199,204} Muller³⁴³ reported that the fungus may suppress cyst nematodes in some sugar beet fields in Germany. High numbers and percentages of *C. xenoplax* parasitized by *H. rhossiliensis* were found in some California peach orchards.¹⁹⁸ Jaffee and Muldoon²⁰⁰ demonstrated that this fungus suppressed penetration of cabbage roots by *H. schachtii*.

Timper and Brodie⁴⁷⁰ reported that *H. rhossiliensis* reduced root penetration by *Pratylenchus penetrans* and suppressed nematode populations 60 days after inoculation. Results obtained by Tedford et al.,⁴⁶⁵ however, indicated that long-term interactions between populations of *H. rhossiliensis* and cyst or root-knot nematodes will not result in biological control. Parasitism of nematodes by *H. rhossiliensis* is dependent on nematode density.¹⁹⁶ Since the fungus is a poor soil competitor, local populations of the fungus may go extinct unless supplied with some minimum number of nematodes (the host threshold density); thus, natural epidemics of this fungus among populations of nematodes develops slowly and only after long periods of high host densities.^{196,205} Some spores of *H. rhossiliensis* died shortly after sporulation and others were viable and virulent for at least 200 days.²⁰² Transmission of spores was greater in loamy sand than in coarse sand.²⁰² The percentage of nematodes parasitized by the fungus was dependent on the number of *C. xenoplax*/100 cm³ soil;¹⁹⁸ but the slope was relatively shallow and the variation in percentage parasitism was large, suggesting that *H. rhossiliensis* is a weak regulator of *C. xenoplax* population densities.¹⁹⁸ Studies on formulations of this fungus for delivery to soil are underway.²⁷¹

Factors affecting efficacy of biological control agents

It is important that information be developed on the modes of action, ecology, and epidemiology of nematode antagonistic fungi. Some reviews^{240,241} have addressed factors, such as soil temperature, moisture, texture, and microflora. The nematode target should be considered when a fungus is used as a biological control agent.^{240,241,521} Facultative parasites of eggs and females, such as *V. chlamydosporium*, may be effective for some

cyst nematodes.²³⁶ Nematode eggs are more susceptible when they are immature than when they contain second-stage juveniles.¹⁹⁵ It is important that fungi infect the nematode soon after the female or egg mass is present on the root surface. A successful parasite of eggs of sedentary endoparasitic nematodes should prove to be a good root colonizer. This is especially important for root-knot nematodes because the females and a large portion of gelatinous matrices containing eggs remain embedded in the root. Only a good root colonizer, such as *P. lilacinus*,³⁵ may attack young females and the embedded egg masses. Fungi that infect mobile stages of nematodes by producing traps or adhesive spores may be more effective against ectoparasitic nematodes.¹³¹

Fecundity of the nematode and the rate of embryonic development are likely to affect the level of control.²⁴⁰ Inoculum level and application time are important factors affecting the efficacy of *P. lilacinus* against *M. incognita*³⁴ and *M. arenaria*.¹⁶⁵ Some efforts have been made to reduce the effective dosage rate.⁴⁰ Fungal nutritional carriers are also an important factor.³⁷ Fungi grown in liquid culture and formulated in sodium alginate granules need an energy source before they become established in soil. However, no such energy source was necessary for inoculum grown on solid media.²⁷¹ Temperature requirements differ among or within species.³⁶ *Verticillium suchlasporium* adapted more to low temperatures than did *V. chlamydosporium*, suggesting that *V. suchlasporium* might have greater potential as a biological control agent in areas with a colder climate. Conversely, *V. chlamydosporium* might perform better under warmer conditions.⁸⁹ Soil texture affects on the proliferation and survival of fungi in soil.²³⁸ In general, *V. chlamydosporium* multiplied more rapidly in organic soil than in mineral

soil.²⁴⁰ The best control of *M. incognita* by the fungal formulation R 350 was obtained in soil containing more than 2% organic matter and near neutral pH.³⁷⁵ Control of *M. incognita* with *V. chlamydosporium* was greater in well aerated soil.¹⁰²

Some Methods for Study on Fungal Antagonists of Nematodes

Methods for isolating nematophagous fungi from soil or nematodes

Isolation of predators and endoparasites of vermiform nematodes. The common methods for isolation of trapping and endoparasitic fungi from vermiform nematodes include soil sprinkle,^{18,173,175} Baermann funnel,^{16,18,173} and differential-centrifugation^{16,18,173} techniques. Both soil sprinkle and Baermann funnel techniques are efficient methods for isolating predator and endoparasitic fungi.¹³ When the sprinkle technique is used, numerous small replicated samples are needed to obtain all the species that can be isolated.¹² The Baermann funnel technique was more effective in isolating endoparasitic nematophagous fungi than the sprinkle method.¹⁷¹ Nicolay and Sikora³⁵⁰ improved the Baermann funnel and differential-centrifugation techniques. Persmark et al.³⁸⁰ found that the largest number of nematodes infected by endoparasitic fungi was obtained with centrifugal flotation with silica⁶⁸ and the Seinhorst elutriation method,⁴²⁴ when compared with Cobb's decanting and sieving method,⁵⁸ the Whitehead direct method (Whitehead tray),⁵⁰⁴ and centrifugal flotation with sucrose²²⁵ and MgSO₄.¹⁸⁸

Isolation of parasites of eggs and sedentary females. The methods used for isolating fungi from nematode eggs and sedentary females or cyst are relatively simple. Usually nematode eggs are spread on the surface of water agar containing one or more

antibacterial antibiotics.^{161,354,446,476} Water agar (1%) or peptone dextrose agar (1%) containing 1,000 μg of Triton-X 100, 50 μg of aureomycin and 50 μg of rose bengal per ml of medium were used to estimate fungal infection of eggs.²⁸⁸ The additives can inhibit bacterial growth and restrict fungal growth so that individual colonies are countable, and the fungi can sporulate and be identified directly on the plates. Females and cysts are usually surface sterilized and placed on water agar containing antibacterial antibiotics.^{45,157} Subcultures on various media are usually made for identification. Crump⁷³ compared methods for isolation of fungi from females. Spreading individual females across plates containing agar and taking three sub-cultures rather than a single sub-culture from one intact female resulted in up to three fungal species being isolated from some females. Crump⁷⁴ also developed a method whereby females and cysts did not require surface-sterilization. The nematodes were placed 0.5 mm from the edge on a sterile coverslip placed on water agar. The fungi grew from females onto the water agar. Culbreath et al.⁸⁴ developed a method for estimating the level of fungal colonization of nematode eggs in soil and for isolation of those fungi. Agar discs containing nematode eggs were buried in soil and the fungal invasion of the eggs was recorded. Drying of soil reduced recovery of fungi from cyst nematode eggs.²⁵⁰ Mitchell et al.³¹⁶ developed a semi-selective medium for the isolation of the nematode-egg parasitic fungus, *P. lilacinus*. Kerry et al.²⁴⁹ also described selective media for isolation of *V. chlamydosporium* from soil.

Methods for quantifying nematophagous fungi

Methods to quantify nematophagous fungi in soil have been developed, but they

do not always give satisfactory results. Dackman et al.⁹¹ developed a method to quantify predatory and endoparasitic nematophagous fungi in soil. Mankau²⁹⁸ described a method of semi-quantitative enumeration and observation of parasites and predators of soil nematodes, which could also be applied for nematophagous fungi. The thick-walled resting spores are relatively easy to separate from soil and quantify. Crump and Kerry⁷⁹ described a method for extracting resting spores of *N. gynophila* and *V. chlamydosporium* from soil. The soil sample is wet sieved and the spores separated by centrifugation in a high specific gravity solution of magnesium sulphate (sp. g. 1.30, concentration 650 g of anhydrous MgSO₄/liter of solution). Selective media have been developed for quantifying *P. lilacinus* and *V. chlamydosporium* in soil.^{249,316} The level of activities of facultative parasites of nematode eggs and sedentary females can be determined in frequency of colonization of eggs, females, or cysts. Methods²⁸⁸ have been developed for isolating total parasitic fungi of eggs and females. The semi-selective medium³¹⁶ is useful for detecting *P. lilacinus* in nematode eggs and sedentary females.

Methods for evaluating nematode suppressive soil

Treatment of soil with fungicides, such as captafol,^{74,187} mancozeb and iprodione,³⁰⁹ benlate,³⁸³ tachigaren,³⁸³ and formalin,^{247,383} has been used in the evaluation of nematode suppressive soil. Irradiation has also been used to detect suppression of nematode by nematophagous fungi in soil.¹⁵² Microwave heating of soil may be useful to kill soilborne fungi with only little effect on prokaryotic organisms.¹³⁷ Subjecting soil to autoclaving or steam heating is also useful for killing soilborne fungi.³⁴¹

Crump⁷⁴ used triangular-shaped observation chambers with removable sides in order to observe the infection by fungi of developing cyst nematodes. A soil fraction technique was described for the estimation of activity of egg-parasitic fungi in field soil.³⁵¹ The cysts are extracted from soil, the eggs and fungi released by crushing, and the cyst contents reincorporated into the original sample. The soil is placed in plastic pot 7 cm in diameter and planted to nematode-host plants.³⁵¹ The fungal parasitism of newly formed cysts was determined.²⁴³ Perry³⁷⁹ proposed a model for estimating the effects of a fungal parasite (*N. gynophila*) on densities of *H. avenae*. The model predicted population decline and attainment of low stage equilibrium densities and a good fit to certain field data was found.

Methods for culture, maintenance and preservation of fungal cultures

Saprophytic fungi can be cultured on potato dextrose agar or cornmeal agar. Most obligate parasitic fungi are difficult to grow in pure culture and must be cultured on the nematode host; however, *N. gynophila* has been cultured on weak brain-heart infusion agar and potato dextrose agar.¹⁶⁷ The loss of virulence must be considered before testing fungi as biological control agents.^{497,525} Storage on dry agar slants under mineral oil or in water are among several methods that have been suggested for the maintenance of nematophagous fungi.^{227,448} The maintenance of virulence in nematophagous fungi also may be ensured by cryopreservation of subcultures soon after isolation from the natural environment.⁵²⁵

The Soybean Cyst Nematode

Distribution and Importance

The 'yellow dwarf' disease of soybean (*Glycine max* (L.) Merr.) is caused by the soybean cyst nematode (SCN), *H. glycines*. Although it is generally documented that the first report of the SCN came from Japan in 1915,¹⁹¹ more recent documentation reported the nematode disease was recognized as early as 1880 in Heilongjiang Province, China.³⁵⁶ The nematode currently infects soybean in Japan, China, Korea, Indonesia, Canada, the United States, Colombia, countries within the former Soviet Union,³⁹⁰ and Brazil.³⁰⁸

The SCN is the most important soilborne pathogen of soybean.¹⁰⁹ The nematode has become disseminated widely in the two major soybean producing countries, the United States and China, and will likely become widely disseminated in Brazil. Although it is difficult to get an accurate estimation of yield suppression by SCN, this nematode has been reported to destroy entire fields in China.⁵⁰ The overall loss in 15 southern states of the United States during 1979-82 was estimated at 2-4%.⁵¹¹ Recent reports from 12 states in the north central region of the United States estimated losses of 1.32 million tons (\$267 million) yearly during 1989-91.¹⁰⁹

Biology

Heterodera glycines is a sedentary endoparasite. The developmental stages include egg, four juvenile stages, male, and female. Following embryogenesis, the first stage juvenile molts to form a second stage juvenile. The second-stage juvenile hatches

and becomes the infective stage. The hatched juveniles move through the soil pores in a film of water surrounding soil particles. They penetrate host roots, generally well behind the zone of differentiation. After penetration, the nematodes will establish a feeding site (syncytium) in vascular tissue. The nematode molts three more times, evolving through the third and fourth stages before becoming males and females. The females are lemon-shaped, whereas males remain vermiform. The female body, when fully developed, lies outside of roots and can be seen with the naked eye. The nematode is amphimictic, and after mating the female exudes a gelatinous matrix into which it lays eggs. After a short period the eggs are retained inside the body. The saccate, lemon-shaped female changes color from white, to yellow, to tan. When a female dies, the female body undergoes a tanning process that induces polyphenol-oxidase activities which result in a cyst. The cyst, which at this stage may be filled with up to 500 eggs, may serve to protect eggs from damage by environmental stresses. Some eggs within the cysts may survive for more than 11 years.⁴²⁰ Diapause of eggs may occur in response to declining soil temperature late in the autumn of the soybean growing season,^{190,401,419} and trehalose accumulation in response to decline of temperature is involved in the diapause induction.⁵¹⁶ The optimal temperature is about 24 C for hatching, 28 C for root penetration, and 28-31 C for development; little or no development takes place at or below 15 C or above 30 C.^{190,420,434}

Variation among populations of *H. glycines* has been demonstrated through various phenotypic and genotypic analyses.³⁴⁸ Four differential cultivars were used to determine races of *H. glycines*.¹⁶⁴ According to this scheme, there are 16 possible

combinations and thus 16 potential races.³⁸⁹ At least 12 races have been reported.³⁸⁹ The typical disease symptoms of soybean induced by *H. glycines* include chlorosis and stunting, from which the disease was named. The nematode inhibits root nodulation and nitrogen fixation; thus, leaves of severely infected plants become yellow-colored. The yellow patches are randomly distributed in the field. Severely infected plants may die before florescence. Root symptoms included necrosis, reduced root system, reduced nodule formation, and broken root tips. Signs of white and yellow females, and brown cysts on root surfaces usually can be seen.

Management

Crop rotation is an effective and practical means of managing *H. glycines* because few crops are susceptible.⁴²⁰ Planting a nonhost crop in a field for 1-3 years can reduce the SCN population level below the damage threshold.⁵¹² Some commercial cultivars that are resistant to SCN can be used efficiently for management. A cropping sequence with susceptible and resistant cultivars and nonhosts may be a good combination for management of the SCN. Crop rotation may not be acceptable in some instances because of limited land availability and market demands. Continuing to plant resistant cultivars may induce race shifts, and eventually resistance breaking races will develop.⁵¹² Furthermore, planting a resistant cultivar may produce 5-10% less yield than would occur with susceptible cultivars in absence of the nematode, because agronomic characteristics of resistant cultivars are often inferior to those of susceptible cultivars.³⁵⁶ Certain nematicides, such as aldicarb, are effective in controlling the SCN. But no

nematicide has been used widely because of high costs compared with the economic value of soybean. Biological control of the SCN appears to have some potential; thus, it is an area that is receiving much attention. Many fungi have been isolated from the nematode (Table 1-2), and a bacterial parasite, *Pasteuria nishizawae* Sayre, Wergin, Schmidt & Starr,⁴¹⁷ has been reported in Japan.³⁵⁵ A species of *Pasteuria* also has been found on SCN in Korea,²⁵⁷ and just recently, a *Pasteuria* sp. was found on SCN in the United States.³⁵⁷ Some fungi have been tested for their efficacy in control of the nematode in the greenhouse^{160,256,257,311,388} and in the field,³⁴⁹ but none have been accepted for practical field use.

Objectives of The Research Project

Nematologists are continuing to seek out biological control agents for management of nematodes. The ecological history of fungi associated with nematode females, eggs and cysts, as well as knowledge of their species composition and their modes of infection are important for selecting and developing an efficient biological control agent. This basic information is also important for improving the management of soil mycoflora so that it becomes more suppressive to nematodes.

A field located at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida was infested with SCN obtained from another agronomy field located on the University of Florida campus in Gainesville. The SCN population developed poorly, suggesting that the soil could be suppressive to this nematode.¹⁰⁶ The objectives of this research project were: 1) to examine the species and frequency of fungi

colonizing different developmental stages of SCN under Florida conditions; 2) to evaluate the influence of selected soil mycoflora on population development of *H. glycines* in a greenhouse bioassay; 3) to determine the pathogenicity of fungi isolated from cysts and eggs of *H. glycines*; and 4) to investigate the mode of infection of these fungi on cysts with the aid of a light microscope and scanning and transmission electron microscopy.

CHAPTER 2 IDENTIFICATION AND DESCRIPTION OF FUNGI FROM CYSTS AND EGGS OF *HETERODERA GLYCINES*

Introduction

Nematophagous fungi have been isolated from many divergent groups of nematodes, and many of these fungi were isolated from the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe (see Chapter 1). Cysts are unique substrate for the soilborne fungi. Several new species have been described from the cysts of SCN.^{42,44,325,336} Extensive studies have shown a taxonomically diverse mycoflora in different locations in the United States,^{45,157,158,326,328} but the most common species are similar among locations. The common genera include *Exophiala*, *Fusarium*, *Gliocladium*, *Neocosmospora*, *Paecilomyces*, *Paraphoma*, *Phoma*, *Stagonospora*, and *Verticillium*.^{45,157,158,326,328} This group of "opportunistic fungi" has the greatest potential as biological control agents of cyst nematodes because they are well-adapted to compete in agricultural soil.³³¹

In this project, the species and frequency of fungi colonizing young white females, older yellow females, and brown cysts of *H. glycines* were investigated on soybean in a Florida soybean field. The fungal species and frequencies of colonization of *H. glycines* eggs were also determined in a greenhouse study. In this chapter the identities and characteristics of these fungi are described. Their frequencies of colonizing

the females, cysts and eggs, and ecological aspects will be presented in Chapters 3 and 4.

Materials and Methods

Fungi were isolated from white and yellow females, brown cysts, and eggs of *H. glycines*. Most cultures of fungi were maintained at room temperature on Difco corn meal agar (CMA). However, potato dextrose agar (PDA), malt extract agar (MEA), and oat meal agar (OMA) (all products of Difco Laboratories, Detroit, MI) were also used to culture certain species of fungi. Features of fungal colonies, including growth rate and measurements of reproductive organs, were recorded for identification. Slides for measurements and morphological observations were generally prepared from the fungal cultures mounted in water or lactophenol. Slide cultures were made and semi-permanent slides were prepared³⁸⁶ to observe the conidiogenous cell, conidiophore, and type of conidiogenesis of some species. Some fungal colonies were not identified because of the lack of sporulation. All observations and measurements were made with the light microscope.

Identities and Description

Chaetomium cochliodes Pall. (= *C. globosum* Kunze). North American Flora 3:61. 1910.

The following characteristics, which conform to those described for *C. cochliodes* by Carris and Glawe,⁴³ were observed for isolate N405. One-week-old colony on CMA

at 23-24 C was 4.4-4.9 cm in diameter, yellowish white, woolly, with irregular borders; reverse side yellowish white. Perithecia formed on the agar surface, green, subglobose, with dark brown peridia of *textura intricata* to *epidermoidea*, covered with perithecial hairs and rhizoidal hyphae, $112-212 \times 112-190 \mu\text{m}$. Perithecial hair dark brown, pale brown towards apices, with denticles, septate, straight at the beginning, become coiled, $2.5-3.3 \mu\text{m}$ in diameter. Asci clavate, thin-walled, eight-spored, deliquescent, $25.2-28.2 \times 12.6-15.1 \mu\text{m}$. Ascospores extruded from the perithecia in cirri, light brown to median brown, smooth, one-celled, with one apical germ pore, subglobose to widely ellipsoid, bilaterally symmetrical, frequently collapsing at maturity, $7.8-10.0 \times 3.3-3.6 \mu\text{m}$.

Curvularia lunata (Wakker) Boedijn. Bulletin du Jardin Botanique de Buitenzorg Series 3, 12:123-127. 1933.

The following characteristics, which conform to those described for *C. lunata* by Ellis,¹³² were observed for isolate I42. One-week-old colony on CMA at 23-24 C covered the agar plate completely, white, with sparse aerial mycelium; reverse side uncolored. Conidiophores brown, unbranched, septate, of variable length, geniculate towards the tip. Conidia boat-shaped, brown, four-celled, with the third cell from base of conidia conspicuously larger and broader than the others, curved or sometimes straight, each with a subhyaline, rounded apical cell, and subhyaline, somewhat obconical basal cell, which bears a scar indicating the point of attachment to the conidiophore, $17.6-24 \times 8.1-12.6 \mu\text{m}$.

Dictyochaeta coffeae (Maggi & Persiani) Cabello & Arambarri (= *Codinaea coffeae* Maggi & Persiani). *Mycotaxon* 20:251. 1984.

The following characteristics, which generally conform to those described for *D. coffeae* by Maggi and Persiani,²⁹⁵ were observed for isolate P32. One-week-old colony on CMA at 23-24 °C was 1.9 cm in diameter. Two-week-old colony on CMA at 23-24 °C was 4.6 cm in diameter, green white to light green gray, cottony, with effuse border. Reverse side of colony uncolored to light green gray. Conidiophores macronematous, brown, straight or slightly curved, smooth, septate, terminal or lateral, frequently with bulbous cell at base, $55-81 \times 3-4 \mu\text{m}$. Conidiogenous cells monophialidic or, frequently, polyphialidic, integrated, terminal or intercalary, bearing one (or occasionally two) conidiogenous locus, at which a distinct, flaring, cupulate, collarette is evident. Conidia hyaline, smooth, suballantoid, to allantoid, one-celled, with none to two guttules, $7.6-15.1 \times 2.8-4.5 \mu\text{m}$; setulae present, $3.5-10.1 \mu\text{m}$ at each end, or occasionally without setulae. Sclerotium-like structures were observed on CMA.

Maggi and Persiani²⁹⁵ described *Codinaea coffeae*, and Cabello and Arambarri⁷ transferred it to the genus *Dictyochaeta*. P32 can be identified to *Dictyochaeta coffeae* (Maggi and Persiani) according to the key to species of *Dictyochaeta* and *Codinaea*.²⁷⁰ However, some sclerotium-like structures that were not reported by Maggi and Persiani²⁹⁵ were also observed on CMA.

Dictyochaeta heteroderæ (Morgan-Jones) Carris & Glawe (= *Codinaea heteroderæ* Morgan-Jones). *Mycotaxon* 33:23. 1988.

The following characteristics, which generally conform to those described for *D. heteroderæ* by Morgan-Jones,³²⁵ were observed for isolate N3. One-week-old colony on CMA at 23-24 C was white and 2.8 cm in diameter; 2-week-old colony on CMA at 23-24 C was 5.2 cm in diameter, greenish white to light greenish gray, cottony, with effuse, uneven border, and reverse side uncolored to light green gray. Conidiophores macronematous, brown, straight or slightly curved, smooth, septate, terminal or lateral, frequently with bulbous cell at base, $25-163 \times 4.5-5 \mu\text{m}$. Conidiogenous cells monophialidic or, frequently, polyphialidic, integrated, terminal or intercalary, bearing one (or occasionally two) conidiogenous locus, at which a distinct, flaring, cupulate collarette is evident. Conidia hyaline, smooth, suballantoid, to allantoid, one-celled, $10.1-17.6 \times 2.8-5 \mu\text{m}$; setulae usually absent, occasionally with short ($0.5-3 \mu\text{m}$) setulae at one end or both ends.

Morgan-Jones³²⁵ described a new species of *Codinaea* (*C. heteroderæ*) isolated from cysts of *H. glycines*.¹⁵⁷ Carris and Glawe⁴³ proposed a new combination of *Dictyochaeta heteroderæ* (Morgan-Jones) Carris & Glawe for this species. The spores of isolates of this species observed in this study occasionally had setulae; colonies on CMA were white to light green gray. Thus these isolates were considered variations within species of *D. heteroderæ*. This species differs from *D. coffeae* in that spores of *D. coffeae* have long setulae.

Drechslera fugax (Wall.) Shoemaker. Canadian Journal of Botany 36:765. 1958.

The following characteristics, which conform to those described for *D. fugax* by Ellis,¹³² were observed for isolate I114. One-week-old colony on CMA at 23-24 C covered the agar plate completely; colonies white, with reverse sides uncolored. Conidiophore brown, producing conidia through an apical pore and forming a new apex by growth of the subterminal region, usually geniculate. Conidia straight, brown, with thick wall, seven to ten-celled, $65.5-88.2 \times 17.6-20.3 \mu\text{m}$.

Exophiala pisciphila McGinnis & Ajello. Mycologia 66:518. 1974.

The following characteristics, which conform to those described for *E. pisciphila* by McGinnis and Ajello³⁰⁵ and by de Hoog and Hermanides-Nijhof,⁹⁹ were observed for isolates N77, N139, and S199. One-week-old colony on CMA at 23-24 C was 1 cm in diameter, deep gray olivaceous, woolly to appressed, with even border; reverse side of colony deep gray olivaceous. Three-week-old colony on CMA at 23-24 C was 3.3 cm in diameter, aerial mycelium sparse. Hyphae 2-3 μm in diameter. Conidiogenous cells enteroblastic, hyaline, smooth, single or aggregated; intercalary, lateral, or terminal; proliferating percurrently; with small neck with frill or annellation (usually not distinct); $5.5-8.7 \times 3-3.5 \mu\text{m}$. Conidia hyaline, smooth, ellipsoidal to obovoid, with truncate base, one-celled, $3.4-7.5 \times 1.7-3.9 \mu\text{m}$.

Fusarium equiseti (Corda) Sacc. *sensu* Gordon. Canadian Journal of Botany 30:225. 1952.

The following characteristics, which conform to those described for *F. equiseti* by Booth,²⁹ were observed for isolate N399. One-week-old colony on CMA at 23-24 C was 7.1 cm in diameter, white to yellow white, woolly, with effuse border; reverse side of colony white. Colony on PDA gray yellow to yellowish gray (peach), cottony; reverse side brownish yellow to light brown. Conidiophores simple or branched, single (or dense sporodochium-like clusters). Conidiogenous cells phialidic, hyaline, smooth, narrowly awl-shaped, straight or curved. Microconidia absent. Macroconidia variable in size, four- to eight-celled, falcate, strongly curved, basal cell distinctly foot-shaped, apical cell elongated, $15.1-45.3 \times 2.3-4.6 \mu\text{m}$. Chlamydospores globose, $6.3-10 \mu\text{m}$ in diameter, intercalary, solitary, in chains or clumps.

Fusarium oxysporum Schlecht. American Journal of Botany 27:66. 1940.

The following characteristics, which conform to those described for *F. oxysporum* by Booth,²⁹ were observed for isolates N21, N37, and N152. One-week-old colony on CMA at 23-24 C was 7.6 cm in diameter, white to light pink, with sparse aerial mycelium and effuse border; reverse side of colony uncolored. Four-day-old colony on PDA at 23-24 C was 4 cm in diameter, white to purple, cottony, with effuse border, reverse side of colony pale white to violet pale purple. Conidiogenous cells phialidic, hyaline, smooth, cylindrical to doliiform; arising laterally from aerial or appressed hyphae. Microconidia hyaline, smooth, ellipsoidal to cylindrical, one- to two-celled, 3.8-

15.1 $\times$ 2.5-3 μ m. Macroconidia hyaline, smooth, fusiform, with foot cell and curved ends, three- to five-celled, 18-28 $\times$ 3-4 μ m. Chlamydospores hyaline to subhyaline, smooth or roughened, globose, one-celled, single or in short chains, terminal or intercalary, 6.3-8.8 μ m in diameter.

Fusarium semitectum Berk. & Rav. Grevillea 3:88. 1875.

The following characteristics, which generally conform to those described for *F. semitectum* by Booth,²⁹ were observed for isolate H12. Four-day-old colony on PDA at 23-24 C was 3.6 cm in diameter, white to puff brown, cottony, with uneven border; reverse side of colony buff brown. Two-week-old colony on PDA at 23-24 C was 6.5 cm in diameter, pale peach to buff brown; reverse side of colony buff brown. Microconidia absent. Macroconidia formed on simple conidiogenous cells or from well developed conidiophore. Conidiogenous cells cylindrical, polyblastic, 7.6-25.2 $\times$ 2.5-3.0 μ m. Macroconidia vary in size, one- to five-septated, curved, with wedge-shaped basal cell, 15.1-37.8 $\times$ 2.3-3.0 μ m. Chlamydospores sparse, globose, intercalary, formed singly or in chains.

The growth rate of H12 was less than that of *F. semitectum* described previously,²⁹ which was 6.1 cm in diameter on PDA at 25 C for 4 days. The color and general appearance of the culture of H12 is similar to that of N399 (*F. equiseti*).

Fusarium solani (Mart.) Sacc. Michelia 2:296. 1881.

The following characteristics, which conform to those described for *F. solani* by Booth,²⁹ were observed for isolates N9 and N13. One-week-old colony on CMA at 23-

24 C was 6.6 cm in diameter, white to yellow white, woolly, with even border; reverse side of colony yellow white. Four-day-old colony on PDA at 23-24 C was 3.5 cm in diameter, white to yellow white, cottony, with effuse border; reverse side of colony pale yellow. Conidiogenous cells phialidic, hyaline, smooth, cylindrical, tapering towards apices, forming periclinal thickenings and collarettes, single or in sporodochia. Conidia of two types: microconidia hyaline, smooth, ellipsoid to cylindrical, one- to two-celled, forming globose heads $5-20 \times 2.5-6.3 \mu\text{m}$; macroconidia hyaline, smooth, cylindrical, with tapering and curved ends, lacking well-defined foot cell, three- to five-celled, $25.2-37.8 \times 4.5-7.8 \mu\text{m}$. Chlamydospore hyaline, smooth, globose, single or in pairs, terminal or intercalary, $5.8 \mu\text{m}$ in diameter.

Gliocladium catenulatum Gilm. & Abbott. Iowa State Journal of Science 1:303. 1927.

The following characteristics, which conform to those described for *G. catenulatum* by Carris and Glawe,⁴³ were observed for isolate N179. One-week-old colony on CMA at 23-24 C was 4.2 cm in diameter, white to greenish white, cottony, with even or effuse border; reverse side of colony pale yellow. Conidiophores macronematous, hyaline, smooth, cylindrical, branching verticillately (primary) or penicillately (secondary), $27.7-156.2 \times 2.5-3.8 \mu\text{m}$. Primary conidiogenous cells phialidic, hyaline, smooth, cylindrical, tapering, $20.2-35.3 \times 1.3-2.5 \mu\text{m}$. Secondary conidiogenous cells phialidic, hyaline, smooth, awl-shaped; $10.1-20.2 \times 2.5-3.5 \mu\text{m}$. Conidia hyaline, green in mass, smooth, asymmetrically ellipsoidal, in globose heads, one-celled, $3.9-8.9 \times 2.0-3.5 \mu\text{m}$.

Gonytrichum macrocladum (Sacc.) Hughes. Transaction British Mycological Society 34:565. 1951.

The following characteristics, which conform to those described for *G. macrocladum* by Carris and Glawe,⁴³ were observed for isolate P146-1. Two-week-old colony on CMA at 24 C was 2 cm in diameter, gray to brown, woolly, with even border; reverse side of colony white to gray. Conidiophores macronematous, up to nine-septate, dark brown, paler towards apex, smooth, cylindrical, tapering, $17.6-151 \times 2.5-4.5 \mu\text{m}$. Conidiogenous cells phialidic, pale brown, awl-shaped, straight to slightly curved, with collarette, terminal or borne in whorls, encircling main axis of conidiophore, $10-17.6 \times 2.5-4 \mu\text{m}$. Conidia subhyaline to pale brown, smooth, ellipsoidal to obovoid, one-celled, $3.3-4.4 \times 2-2.9 \mu\text{m}$, in masses at apices of conidiogenous cells.

Helicomyces sp.

Isolate A4 had the following distinguishing characteristics: conidiophore simple, hyaline; conidia hyaline, septate, filamentous, coiled in one plane.

Humicola sp. 1.

The following characteristics, which conform to those described for the genus *Humicola* by Ellis,¹³² were observed for isolate E1-4. One-week-old colony on CMA at 24 C was 6.2 cm in diameter, white, with sparse aerial mycelium and even border; reverse side of colony white. One-week-old colony on PDA at 24 C was 4.5 cm in diameter, yellowish orange, white toward border, with mycelium mostly immersed and

even border; reverse side of colony same colored; 1-week-old colony on V-8 agar 4.5 cm in diameter, similar in feature as on PDA. Mycelium septate, 1-2.5 μm in diameter. No distinct conidiophore. Chlamydospores (aleuriospores) abundant, formed in medium or on aerial mycelium, globose, one-celled, 12.6-20.2 μm in diameter, borne laterally or terminally on mycelium. No thin-walled conidium was observed.

Humicola sp. 2.

The following characteristics, which conform to those described for the genus *Humicola* by Ellis,¹³² were observed for isolates G3 and E3-11. One-week-old colony on CMA at 24 C was 3.1 cm in diameter, white, wooly, with highly erected mycelium and appressed, fan-shaped border; reverse side of colony white. One-week-old colony on MEA at 24 C was 2.6 cm in diameter, white to pale purple, floccose, with fan-shaped border; reverse side of colony pale purple. Mycelium septate, 1-2.5 μm in diameter. No distinct conidiophores. Chlamydospores globose, one-celled, 7.5-13.9 μm in diameter, borne laterally or terminally on mycelium. No thin-walled conidium was observed.

Microsphaeropsis olivacea (Bonord.) Hoehnel. Hedwingia 59:267. 1917.

The following characteristics, which conform to those described for *M. olivacea* by Carris and Glawe,⁴³ were observed for isolate E1-4. One-week-old colony on CMA at 24 C was 3.7 cm in diameter, olivaceous at center (2.1 cm in diameter), white toward border, floccose, with even border; reverse side of colony same colored. One-week-old

colony on MEA at 24 C was 2.7-2.9 cm in diameter, cream, floccose, with even border; reverse side of colony black at center (0.8 cm in diameter), cream, olivaceous white toward border. Pycnidia globose, brown, setose, with one ostiole of 35 μm in diameter; wall consists of four-five outer layers of dark brown, thick-walled cells, and one or two inner layers of hyaline, thin-walled cells; 170-370 μm in diameter. Conidiogenous cell phialidic, hyaline, dolliform, sometimes proliferate. Conidia oval to ellipsoid, light brown, brown in mass, with none to two guttules, $3.9-4.4 \times 2.2-2.6 \mu\text{m}$.

Myrothecium sp.

The following characteristics, which conform to those described for the genus *Myrothecium* by Ellis,¹³² were observed for isolate S166. Dark-green to black sporodochium formed on CMA. Spore mass viscous, green when young, later becoming hard and black. Conidiophore erected in a compact layer, septate, hyaline, branched, ultimately surmounted by verticil of phialides. Phialospores hyaline, ovoid, gathering in slimy masses

Neocosmospora vasinfecta E. F. Smith. United States Department of Agriculture Bulletin 17:45. 1899.

The following characteristics, which conform to those described for *N. vasinfecta* by Cannon and Hawksworth³⁸ and by Carris and Glawe,⁴³ were observed for isolates N2, N24, N74, and P31. One-week-old colonies on CMA at 22-24 C was 3.9-5.2 cm in diameter, white to pink white, cottony, mostly appressed, with even border; reverse side

of colony uncolored. Pale yellow perithecia formed on surface of agar, becoming pale orange to reddish brown, obpyriform, glabrous, peridium *textura angularis*, $280-324 \times 235-259 \mu\text{m}$. Asci cylindrical, thin-walled, eight-spored, uniseriate, apices bluntly rounded, $81-88 \times 10-12 \mu\text{m}$. Ascospores subhyaline to pale orange-brown, dark orange to salmon pink in mass, slightly roughened, ovoid to ellipsoid, one-celled, thick-walled, $9.6-12.6 \times 8.1-9.7 \mu\text{m}$.

Anamorph: *Acremonium* sp. Conidiogenous cells phialidic, hyaline, cylindrical, with tapered apices. Conidia hyaline, smooth, ellipsoidal to fusiform, usually one-celled.

According to the 'Key to species of *Neocosmospora*',³⁸ the isolates N2, N24, N74 and P31 can be identified as *N. vastinfecta*. But there are variations of the colony features and the color of perithecia among the isolates.

Paecilomyces inflatus (Burnside) Carmichael (= *Mycelophthora inflata* Burnside).
Canadian Journal of Botany 40:1148. 1962.

The following characteristics were observed for isolate E5-11. Most characteristics of isolate E5-11 conform to those described for *P. inflatus* by Burnside³² and by Samson.⁴¹¹ Two-week-old colony on PDA at 24 C was 3.6 cm in diameter; white; with a concentric, olive-colored, fan-shaped ring; floccose; with even border; reverse side of colony olive to black, yellowish white toward border. Two-week-old colony on MEA at 24 C was 2.0 cm in diameter, pale olive, yellowish white toward border, felt, with even border; reverse side of colony olive, yellowish white toward border. Four-week-old colony on V-8 agar at 24 C was 4.5-6 cm in diameter, pale

yellow, felt, with fan-shaped border; reverse side of colony pale yellow with pale brown concentric ring. Two types of mycelium: hyaline, thin-walled, smooth mycelium, 1.1-4.5 μm in diameter; and brown, roughened mycelium, 2.3-5.1 μm in diameter. Conidiogenous cell phialidic, mostly borne directly on undifferentiated hyphae, some on short base cell, flattened at base or middle, $7.8-12.2 \times 2.8-3.9 \mu\text{m}$; hyaline if borne on hyaline hyphae; light brown to brown if borne on brown hyphae. Conidia lemon-shaped, some subglobose, ovoid, ellipsoid, smooth or slightly roughened, one-celled, $3.9-6.7 \times 2.7-2.9 \mu\text{m}$.

Paecilomyces inflatus was first isolated from abdomen of an adult worker bee and described as *Mycelophthora inflata* Burnside.³² Isolate E5-11 is the first isolate of this fungus from the SCN, and perhaps this constitutes the first report from nematodes. Isolate E5-11 has some differences from the original description for *M. inflata* by Burnside, in that no chlamydospore was observed in E5-11, while abundant chlamydospores were reported by Burnside; additionally the conidia of E5-11 are larger than those in the original description for the fungus ($3.25-3.5 \times 2.5-2.75 \mu\text{m}$). Samson,⁴¹¹ however, described the absence of chlamydospores, conidia of $3-4 \times 2-3 \mu\text{m}$ and the lack of roughen mycelium. The differences between E5-11 and the original description for *M. inflata* is considered to be within the range of natural variations.

Paecilomyces lilacinus (Thom) Samson. Study in Mycology 6:58. 1974.

Most of following characteristics observed for isolates N303-1 and M45-7 conform to those described for *P. lilacinus* by Samson.⁴¹¹ One-week-old colony on CMA

at 23-24 C was 2.8 cm in diameter, pale pink to gray pink, cottony; reverse side of colony uncolored. Four-day-old colony on PDA at 23-24 C was 2 cm in diameter, pale pink, white toward border, cottony, with appressed, even border; reverse side of colony yellowish white. Two-week-old colony on PDA at 24 C was 4.8 cm in diameter, vinaceous at center, yellowish white toward border, floccose, with even border; reverse side of colony yellowish brown to liver brown. Two-week-old colony on MEA at 24 C was 5.6 cm in diameter, white, floccose, with even border; reverse side of colony white. Conidiophore macronematous, hyaline in young culture, becoming light brown to brown in old culture, smooth or roughened, cylindrical, $7-150 \times 2.2-3.2 \mu\text{m}$, borne laterally or terminally on hyphae. Conidiogenous cells phialidic, hyaline, smooth, narrow flask-shaped with tapering necks, $6.0-12.1 \times 2.2-3.0 \mu\text{m}$. Conidia hyaline, purplish pink in mass, smooth, ovoid, borne in chains, $2.3-3.3 \times 1.7-2.3 \mu\text{m}$. Chlamydospore-like structures were observed in one old culture on CMA, globose ($5.0-7.5 \mu\text{m}$ in diameter) to ellipsoid, hyaline.

N303-1 was isolated from a cyst of SCN collected from the soybean field. M45-7 was isolated from an egg mass of root-knot nematode from a tobacco root taken from the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida. Most characters of these isolates were similar to those of *P. lilacinus* described in Samson's review.⁴¹¹ But compared to the Peruvian strain of *P. lilacinus*, M45-7 and N303-1 produced longer conidiophores (up to $150 \mu\text{m}$ in M45-7 vs. $60 \mu\text{m}$ in Peruvian strain) in old cultures; the reverse color of colony M45-7 on PDA is yellowish white to liver brown vs. vivacious to brownish black in the Peruvian strain. The identity of M45-7 was

confirmed by Dr. R. A. Samson in Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The difference between the Peruvian strain and the strain isolated in this study is considered a variation within *P. lilacinus*.

Papulaspora sp. 1.

The following characteristics, which conform to those of the genus *Papulaspora* described by Arx,⁸ were observed for isolate N95. One-week-old colony on CMA at 23-24 C covered plate completely, white, appressed, with immersed mycelium. Numerous black papulaspores forming on immersed hyphae, subglobose to irregular, multi-celled, usually surrounded by hyaline cells. Central cells larger, arising from intercalary cells on hyphae.

Paraphoma radicina (McAlp.) Morgan-Jones & White, Mycotaxon 18:57. 1983.

Morgan-Jones and White³³⁵ established a new genus, *Paraphoma*, to separate *Phoma radicina* from the genus *Phoma* based on the study of an isolate from cysts of *H. glycines*. Although Van der An et al.⁴⁸⁴ treated *Paraphoma* as a section under *Phoma*, *Paraphoma* is accepted as a valid genus in this study.

The following characteristics, which conform to those of *P. radicina* described by Morgan-Jones and White,³³⁵ were observed for isolate N70. One-week-old colony on CMA at 23-24 C was 2.7 cm in diameter, gray greenish yellow to median olivaceous, cottony to appressed, with even border; reverse side of colony gray greenish yellow to median olivaceous. One-week-old colony on PDA was 1.9 cm in diameter, yellowish

brown, felty, with hyphae in marginal zone submerged and even border; reverse side of colony brown. Pycnidia forming after 2 weeks on CMA, median brown to brownish black, globose, with short neck or without neck, covered completely with setae, with ostiole of 10.1-12.6 μm in diameter, peridium *textura angularis*, 145-157 μm in diameter. Pycnidium wall thick, composed of one-two out layers of brown cells and about four inner layers of thin-walled cells. Setae dark brown, smooth, thick-walled, straight or slightly curved, septate, unbranched, 44.5-111.8 $\times$ 2.0-3.3 μm . Conidiogenous cells doliiform or cylindrical. Conidia hyaline, obovate to ellipsoid, guttulate, one-celled, smooth, thin-walled, 3.8-5.0 $\times$ 2.5-3.0 μm . Chlamydospore not observed.

Paraphoma sp.

The following characteristics, which conform to those of the genus *Paraphoma* described by Morgan-Jones and White,³³⁵ were observed for isolate N224. One-week-old colony on CMA at 23-24 C was 2.3 cm in diameter, white to gray reddish brown, cottony, with effuse border; reverse side of colony white to gray reddish brown. Pycnidia formed after 3 weeks, median brown to brownish black, setose, with one or more ostiole, globose to subglobose, single or occasionally aggregated, sometimes confluent, formed superficially on the agar or slightly immersed, 168-415 $\times$ 145-257 μm , with a neck of 89.0-111.8 μm long and 44.7-111.8 μm in diameter. Pycnidial ostioles circular, 12-12.6 μm in diameter. Pycnidial setae usually around neck of pycnidia, dark brown, thick-walled, septate, straight or slightly flexuous, smooth or

verrucose; apices acuminate; 27.7-113.4 μm long, 4.5-5.3 μm wide at base, 2.5 μm at apices. Pycnidial wall thick, with about six layers of pseudoparenchymatous cells. Conidiogenous cells simple, arising from inner wall of pycnidia, lageniform or short cylindrical. Conidia enteroblastic, ellipsoid, aseptate, hyaline, smooth, guttulate, 4.4-5.5 $\times$ 1.3-1.9 μm .

The genus *Paraphoma* can be distinguished from *Pyrenochaeta* by short, simple conidiogenous cells and lacking conidiophores; it can be distinguished from *Phoma* by the presence of setae on the surface of pycnidia.³³⁵ Based on these two characteristics, N244 belongs to *Paraphoma*. However, N244 is different from *Paraphoma radicina*, the type species of the genus, in that N244 possesses pycnidia with more than one ostiole, the width of conidia of N244 is much less than that of *P. radicina*, and the colony color of N244 on CMA is white to gray reddish brown rather than olivaceous. The culture was lost during storage, so no further observation was made.

Periconia macrospinos Lefebvre & Johnson. Mycologia 41: 417. 1949.

The following characteristics, which conforms to those of *P. macrospinos* described by Ellis³² and by Carris and Glawe,⁴³ were observed for isolate N286. Conidiophores macronematous, pale brown to brown, smooth, multiseptate, bearing terminal and lateral branches of conidiogenous cells, 32.7-105.8 $\times$ 6.3-7.1 μm . Conidiogenous cells polyblastic, pale brown, smooth or roughened, globose to obovoid, irregularly branched, 6.3-8.8 $\times$ 5.3-6.3 μm . Conidia dark brown, globose, muriform, with dense aggregations of spines 3.8-7.6 μm long, 10.1-20.2 μm in diameter, formed

in acropetal chains, maturing retrogressively from apex. Chlamydospores subhyaline, smooth, globose 10.1-11.4 μm in diameter to ellipsoidal 5.5-6 $\times$ 2.7-4.0 μm , thin-walled, intercalary, lateral or terminal, single or in chains.

Phoma chrysanthemicola Hollos. Annales historico-naturales Musei Nationalis Hungarici 5:456. 1907.

The following characteristics, which conforms to those of *P. chrysanthemicola* described by Sutton,⁴⁶⁰ were observed for isolate I99. One-week-old colony on CMA at 23-24 C was 4.5 cm in diameter, white to olivaceous grey, floccose, with even border; reverse side of colony white to olivaceous. Pycnidium median brown, glabrous, globose to subglobose, peridium *textura angularis*, 105-187 μm in diameter, with opening 7-10 μm in diameter. Conidiogenous cells, phialidic, doliiform, formed on inner wall of pycnidium. Conidia ellipsoidal, smooth, straight or slightly curved, hyaline, guttulate, thin-walled, one-celled, 5-7.0 $\times$ 1.2-1.7 μm . Chlamydospores multicellular, brown, aggregating to form pseudosclerotia.

Phoma sp. 1.

The following characteristics, which conform to those of the genus *Phoma* described by Sutton,⁴⁶⁰ were observed for isolate II4. One-week-old colony on CMA at 24 C was 6.4 cm in diameter, olivaceous at center, white toward border, floccose, with even border; reverse side of colony same color as the face. One-week-old colony on MEA at 24 C was 3.3 cm in diameter, brown floccose, with even border; reverse side

of colony brown. No crystals formed on MEA. Pycnidium median brown, glabrous, globose to subglobose, frequently with more than one ostiole, peridium *textura angularis*; 112-179 μm in diameter, with opening 8.8 μm in diameter. Conidiogenous cells, phialidic, doliiform, formed on inner wall of pycnidium. Conidia ellipsoidal, smooth, straight or slightly curved, hyaline, biguttulate, thin-walled, one-celled, occasionally with one septum, $5.6.9 \times 2.2-3.3 \mu\text{m}$. Chlamydospores abundant, unicellular, median to dark brown, smooth, intercalary, lateral or terminal, often catenulate, 10.1-17.6 μm in diameter.

I14 is close to *Phoma eupyrena* Sacc., but the conidia of I14 are larger than those of *P. eupyrena* ($3.5-5 \times 1.5-2 \mu\text{m}$). It is also close to *Phoma medicaginis* Malbre. & Roum., but no crystals were formed on MEA and no septate conidia were observed. According to the identification by Dr. B. C. Sutton in International Mycological Institute (IMI), UK, this species does not conform to any known species but is closest to *Phoma betae* Frank. The conidia of I14, however, are narrower than in *P. betae*. A dried, preserved example of this isolate (IMI number 361195) has been placed in the IMI dried reference collection. This species will be described as a new species.

Phoma sp. 2.

The following characteristics, which conform to those of the genus *Phoma* described by Sutton,⁴⁶⁰ were observed for isolate E2-14. One-week-old colony on CMA at 24 C was 7.5 cm in diameter, olivaceous, white toward border, with an even margin and sparse aerial mycelium; reverse side of colony same colored. Two-week-old colony

on CMA at 24 C covered 9-cm-in-diameter petri dish, olivaceous, cottony, with even border; reverse side of colony olivaceous. One-week-old colony on MEA at 24 C was 6.5 cm in diameter, orange, white toward border, floccose, with even border; reverse side of colony same color. One-week-old colony on PDA covered 9-cm-in-diameter petri dish, olivaceous, cottony, with even border; reverse side of colony same color. Hyphae hyaline to light brown, 1-5 μm . Pycnidia formed in 3-day-old colony on CMA at 24 C, globose to subglobose, brown to black, one ostiole or sometimes two-three ostioles of 22-45 μm in diameter, peridium *textura angularis*, usually with some hair-like mycelium on surface of old pycnidia, 71-159 μm in diameter. Conidiogenous cell simple, dolliiform. Conidia ellipsoid, with two guttules, $3.8\text{-}8.8 \times 2\text{-}2.8 \mu\text{m}$. Chlamydospores abundant, globose, 7.6-25.2 μm , terminal or intercalary, borne on terminal mycelium, directly on side of hyphae, on short branch of conidiophore, or formed intercalary in chain.

Together with I14, this *Phoma* isolate was also sent to Dr. Sutton for identification and it does not fit any known species. According to Dr. Sutton's identification report, this fungus is close to *Phoma tropica* Schneider & Boerema, but the conidia are about 2 μm longer and 1 μm wider than those of *P. tropica*. Furthermore, E2-14 produces abundant single-celled chlamydospores that are absent in *Phoma tropica*. A dried preserved example of this isolate (IMI number 361196) has been placed in the IMI dried reference collection. This species will be described as a new species.

Pseudorobillarda sojae Uecker & Kulik. Mycologia 78:450. 1986.

Most of the following characteristics observed for isolate S23 conform to those of *P. sojae* described by Uecker and Kulik⁴⁸² and by Raj.³⁸⁵ One-week-old colony on CMA at 24 C was 2.1 cm in diameter, white, appressed, with even border; reverse side of colony uncolored. Pycnidia formed after 1 month on CMA along the edge of the petri dish, immersed on CMA, superficial on the stems of soybean and wheat, pale brown at first, becoming black later, varying in shape, globose, elongate, or mostly confluent; peridium *textura angularis*. Mycelium hyaline, 1.3-2.5 μ m in diameter. Paraphyses absent. Conidiogenous cell cuboid, smooth, often detached from pycnidium wall and attached to the base of conidia. Conidia cylindrical to fusiform; colorless; smooth; guttulate; $4.2-5.5 \times 13.2-16.5$ μ m; bearing at one end (probably at base) two to six (mostly four), attenuated, flexuous, appendages of 16-18 μ m long.

Uecker and Kulik⁴⁸² described a new species, *Pseudorobillarda sojae*, from soybean stems. The conidiogenesis and morphology of S23 conform exactly with the original description of *P. sojae*.⁴⁸² Isolate S23, however, varies from the original description of *P. sojae* in several characters. This isolate does not sporulate well on CMA, PDA, MEA, OMA, wheat stems and leaves, and soybean stems, leaves and roots, whereas *P. sojae* (isolated by Uecker and Kulik⁴⁸²) produced abundant conidia on PDA and soybean stems. Pycnidia of S23 vary in shape and lack a neck, whereas *P. sojae* was reported to produce globose or depressed globose pycnidia with a neck. Furthermore, no definite ostiole was observed in S23 cultured on the substrates tested, although the definite ostiole is a characteristic of the genus *Pseudorobillarda*.

Pseudorobillarda is close to the genus *Robillarda*. In *Pseudorobillarda* the conidioma bears a definite ostiole, conidium ontogeny is enteroblastic, conidia are uniseptate or rarely triseptate with basal appendages, and the paraphyses are simple or sometimes branched. In *Robillarda* the carbonaceous conidioma lacks a distinct ostiole, conidium ontogeny is holoblastic, the conidia are uniseptate with apical appendages, and paraphyses are lacking.⁴⁸² Despite some similarity to *Robillarda*, S23 is identified as *P. sojiae* because most of the characteristics fit the species. Additional comparative studies with the type culture of *P. sojiae* will be required to determine if it can be separated from *P. sojiae*.

Pyrenochaeta terrestris (Hansen) Gorenz, Walker & Larson. Phytopathology 38:838. 1948.

The following characteristics, which conform to those of *P. terrestris* described by Carris and Glawe,⁴³ were observed for isolates N10, I112, and E1-9. One-week-old colony on CMA at 23-24 C was 4.1 cm in diameter, median red, white to light pink toward border, cottony, with even border; reverse side of colony light pink to red. Pycnidia forming after 1 month, brown to black, with dense hairy hyphae on surface, globose, subglobose, frequently confluent and irregular in shape, with setose neck, papillate to elongate and tortuous; immersed in agar, aggregated, occasionally solitary, with one or more ostioles, $224-313 \times 157-246 \mu\text{m}$. Pycnidial neck $55.9-64.0 \mu\text{m}$. Pycnidial setae dark brown, thick-walled septate, straight or slightly flexuous, smooth or verrucous, apices round, $50.4-146.1 \mu\text{m}$ long, $3.8-5.0 \mu\text{m}$ wide at base and $2.3-3.8$

μm at apices. Pycnidial wall thick, up to 12 layers of cells. Two types of conidiogenous cells were formed: lageniform, short cylindrical, elongate cylindrical, arising directly from inner wall of pycnidia; and integrated on septate conidiophores. Conidiophores $8.9\text{--}12.1 \times 2.2\text{--}2.8 \mu\text{m}$. Conidiogenous cells $3.3\text{--}4.4 \times 2.8\text{--}3.3 \mu\text{m}$ (doliiform) or $5.5\text{--}7.7 \times 1.4\text{--}2.3 \mu\text{m}$ (cylindrical). Conidia enteroblastic, ellipsoid, aseptate, hyaline, smooth, guttulate, $3.9\text{--}5.5 \times 1.1\text{--}2.6 \mu\text{m}$.

The elongated septate conidiophores are characteristic of *Pyrenochaeta*. Two types of conidiogenous cells were formed in pycnidia of isolates collected in this study: simple, obpyriform phialides; and cylindrical, elongate, septate conidiophores with integrated conidiogenous cells. These two types of conidiogenous cells of *Pyrenochaeta* were also observed by Carris and Glawe.⁴³

Ramichloridium subulatum de Hoog. Studies in Mycology 15:83. 1977.

The following characteristics, which conform to those of *R. subulatum* described by de Hoog,⁹⁹ were observed for isolate N193. One-week-old colony on CMA at 23-24 C was 1.6 cm in diameter, light gray olive, with appressed mycelium and effuse border; reverse side of colony light gray olive. Three-week-old colony on PDA at 23-24 C was 5.5 cm in diameter, gray olive, farinose due to conidiophore production, with uneven border; reverse side of colony light gray olive. Conidiophores macronematous, polyblastic, median brown, cylindrical, often constricted at base, straight, or curved, septate, $25.2\text{--}75.6 \times 2.5\text{--}3.3 \mu\text{m}$; upper part covered with denticles; arising laterally or terminally from mycelium, single or in clumps. Conidia pale brown, obovoid, base

apiculate, one-celled, some with hyaline perisporium extending beyond spore wall, $3.9-8.6 \times 2.4-3.4 \mu\text{m}$, formed singly, readily dislodged from denticle.

Scolecobasidium terreum Abbott. Mycologia 19:30. 1927.

The following characteristics, which conform to those of *S. terreum* described by Ellis,¹³² were observed for isolate E2-30. One-month-old colony on CMA at 24 C was 3.3 cm in diameter, pale brown, cottony toward center, with even border; reverse side of colony pale brown to olive. Mycelium light brown, $1-3 \mu\text{m}$ in diameter. Conidiogenous cell phialidic, $5.5-7.7 \times 1.7-2.2 \mu\text{m}$. Conidia two-celled, the apical cell end extends in two branches that are nearly vertical with the base cell to become 'T' or 'Y'-shaped, smooth or slightly roughened, $8.9-11.1 \mu\text{m}$ long, $2.2-2.5 \mu\text{m}$ wide at septum, $5-5.5 \mu\text{m}$ wide at apical end. Chlamydospores formed laterally or terminally on mycelium, brown, mostly one-celled, globose, $3.3-5.5 \mu\text{m}$ in diameter. Rarely two- or three-celled conidia were also observed.

Scolecobasidium tricladiatum Matsushima. Microfungi of the Solomon Islands and Papua-New Guinea:52. 1971.

The following characteristics, which conform to those of *S. tricladiatum* described by Matsushima,³⁰³ were observed for isolate E6-3. One-month-old colony on CMA at 24 C was 7.5 cm in diameter, white becoming grayish brown, with sparse aerial mycelium and even border; reverse side of colony same color. Two-week-old colony on PDA at 24 C was 4.4 cm in diameter, black at center, pale yellow toward border,

floccose, with even border; reverse side of colony gray to pale green at center, pale yellow toward border. Conidiogenous cells phialidic, solid, broaden at middle, borne on single hyphae or sporodochia, up to 15.5 μm long; or conidia borne on simple conidiogenous cell. Conidia vary in size and shape, most ellipsoid, some ovoid, clavate or irregular-shaped, one-celled, hyaline, thin-walled, $3.3\text{-}7.7 \times 1.7\text{-}3.3 \mu\text{m}$. Chlamydospores abundant, lemon, pyriform, ellipsoid or irregular in shape, brown, thick-walled, one- or two-celled, or connected in train, $5.5\text{-}13.3 \times 3.3\text{-}5.5 \mu\text{m}$.

Scytalidium lignicola Pesante. Annali della sperimentazione Agraria 11:264. 1957.

The following characteristics, which conform to those of *S. lignicola* described by Ellis,¹³² were observed for isolate I30. One-week-old colony on CMA at 24 C was 7 cm in diameter, pale orange, floccose, with even border; reverse side of colony pale orange. One-week-old colony on PDA at 24 C was 7.8 cm in diameter, orange, floccose, with even border; reverse side of colony orange. Hyphae smooth, hyaline to median brown, 2.5-8.0 μm in diameter. Conidia catenate, separating, smooth, aseptate, sometimes with septa; of two kinds: 1) hyaline, cylindrical, $5.6\text{-}14.5 \times 2.5\text{-}4.2 \mu\text{m}$, or 2) brown thick-walled, ovoid, cylindrical, doliiform, or broadly ellipsoidal, $6.3\text{-}12.1 \times 5\text{-}5.8 \mu\text{m}$.

Stagonospora heteroderae Carris, Glawe & Morgan-Jones. Mycotaxon 29:451. 1987.

The following characteristics, which conform to those of *S. heterodera* described by Carris et al.,⁴⁴ were observed for isolates E2-18, I88, and N6. One-week-old colony

on CMA at 23-24 C was 2.8 cm in diameter, white to green, woolly, with even border; reverse side of colony yellow white, light gray at center. Two-week-old colony on CMA at 23-24 C was 6.1 cm in diameter, green white, light green gray to light pink, zonate, woolly, with even border; reverse side of colony green white, light green gray to light pink, zonate. Pycnidia brown to black, hairy, globose, with a short neck and one or more ostioles about 20.1 μm , 358-391 μm in diameter, immersed or semi-immersed in medium. Conidiogenous cells flask-shaped, cylindrical, straight or tortured, tapering or funnel shaped at top, 11.1-18.8 μm long. Conidiophores absent. Conidia cylindrical to vermiform, hyaline to subhyaline, becoming brown in old culture, three-septated at maturity, with gelatinous caps on one or both ends and truncate base cell, 16.6-25.5 $\times$ 4.4-5.0 μm . Chlamydospores brown in color, one to several cells, intercalary or lateral.

Stagonospora peridium textura angularis. Peridium of isolates in this study *textura intricata* outer layer and *angularis* inner layer. This is considered normal variation within the genus and species. The gelatinous cap on conidia was conspicuous when the conidia is young and stained with cotton blue. This characteristic was not mentioned by Carris et al.,⁴⁴ although it can be seen in illustrations in the original description of the species.

Trichoderma lignorum (Tode) Harz.. Bulletin de la Société Impériale des Naturalistes de Moscou 44:116. 1871.

The following characteristics, which generally conform to those of *T. lignorum* described by Gilman,¹⁵⁶ were observed for isolate P125. One-week-old colony on CMA

at 24 C covered the 9-cm-in-diameter petri dish, white with greenish conidia formed in tufts in ring-like zone close to the edge of the colony, floccose toward border; reverse side of colony uncolored. Conidiophores arise as branches of aerial mycelium, septate, smooth, up to 65 μm long, 5 μm in diameter at the base and 3 μm in diameter near apex, di- or trichotomously branched. Conidiogenous cells phialidic, subhyaline, smooth, obpyriform with constricted bases, lateral or terminal, $5-7.5 \times 2.8-3.5 \mu\text{m}$. Conidia globose, unicellular, smooth, 2-3.3 μm in diameter.

Most characteristics of P125 are similar to those of *T. lignorum* except that the conidia of P125 are a little smaller than those of *T. lignorum* (conidia 3.2-3.8 μm in diameter) described by Gilman.¹⁵⁶

CHAPTER 3
FUNGI ASSOCIATED WITH FEMALES AND CYSTS OF
HETERODERA GLYCINES IN A FLORIDA SOYBEAN FIELD

Introduction

The soybean cyst nematode, *Heterodera glycines* Ichinohe, is one of the most important pathogens of soybean (*Glycine max* (L.) Merr.). It occurs on soybean in most soybean growing countries and regions (See Chapter 1). The costs of applying nematicides to control this pest are usually high, and many nematicides are no longer registered for use on soybean. Therefore, more attention is being given to alternative management tactics, such as crop rotation, cultural methods, plant resistance, and biological control.

Fungal antagonists of plant-pathogenic nematodes have been studied for more than 100 years,^{45,475} and interest in those affecting sedentary endoparasitic nematodes has increased rapidly in recent years.⁴⁴⁷ The swollen females and cysts of cyst nematodes provide unique niches for some soil fungi. In a greenhouse study it was determined that a number of fungi were capable of invading young females and as the females become more exposed in the soil they were increasingly vulnerable to fungi.^{157,158} Some reports revealed a degree of natural control of cyst nematodes by fungi associated with females and eggs.²³⁷

Many fungi have been isolated from the females or cysts of *H. glycines* (Chapter

1, Table 1-2). Extensive studies have shown a taxonomically diverse mycoflora in different locations in the United States,^{45,157,158,326,328} but the most common species are similar among locations. The fungi associated with females and eggs may have great potential as biological control agents of cyst nematodes because these fungi are facultative endoparasites and are well-adapted to compete in agricultural soil.³³¹ Ecological information about fungi associated with nematode eggs, females, and cysts, including knowledge of fungal species composition and frequency, is important for selecting and developing biological control agents for control of cyst nematodes. This basic information is also important for developing the potential of the soil mycoflora to suppress nematodes.

The objective of this study was to examine the species and frequency of fungi colonizing white females, yellow females, and brown cysts of *H. glycines* on soybean in soil in a Florida soybean field.

Materials and Methods

A field located at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida, was used for this study. The soil was an Arredondo fine sand (91% sand, 4.5% silt, 4.5% clay; 1.8% organic matter; pH 5.7).¹⁰⁶ Soybean cyst nematode obtained from another agronomy field located on the University of Florida campus in Gainesville, Florida, was introduced to the site in 1985. The SCN population developed poorly, suggesting that the soil could be suppressive to this nematode.¹⁰⁶

This investigation was conducted during 1991-1992. In 1991, six plots, each with

eight rows and a row spacing of 76 cm and row length of 9.1 m, were used. Cobb and Braxton, two soybean cultivars susceptible to SCN, were planted on 28 May. On 19 June, about 120 soybean plants were taken from each plot by carefully digging up their root systems. Soybean roots were washed with tap water and put in sterilized water. The young white females that contained zero or few eggs and older yellow females were removed with the aid of a stereomicroscope and transferred to sterilized water. On 3 July, about 120 soybean plants with rhizosphere soil were taken from each plot. The roots with attached soil were washed by tap water onto a sieve with 150- μ pore openings. White and yellow females were extracted from the soybean roots as described above. Brown cysts were extracted from the soil and debris remaining on the sieve by a modified sugar-flotation-centrifugation technique²²⁵ with 1.5 kg sucrose/liter of water. On 1 October, the end of the soybean season, samples of 4 kg of soil were taken with a bucket auger (10 cm in diameter) from the soybean rhizosphere 0-20 cm deep from each plot. Brown cysts were extracted from a subsample of about 2 kg of soil from each plot with the technique described above.

On 5 June 1992, 10 rows in each of six plots were planted with the soybean cultivar Cobb in approximately the same sites and with the same row spacing and length as in 1991. On 20 July and 9 September, white and yellow females and brown cysts were extracted with the same procedures as described for the samples of 3 July 1991. On 3 October, brown cysts were extracted by the same procedures used for the samples of 1 October 1991.

The extracted females and cysts were washed with sterilized water, treated with

0.5% NaOCl for 3 minutes, rinsed three times with sterile deionized water, and finally treated with a solution of 100 ppm streptomycin and 50 ppm chlortetracycline. The treated cysts were transferred to water agar and incubated at room temperature (23-24 C). Subcultures were made from fungal mycelium growing from the nematodes after 3-5 days, or after 10-14 days for slow-growing fungi. The isolated fungi were identified (see Chapter 2) and the percentage frequency of white females, yellow females, or brown cysts colonized by each fungal species was recorded on each sampling date.

These frequency data on the fungal communities associated with cysts or females were used to calculate similarity indices. Similarity indices are used in ecology³⁶⁶ to compare the composition of two communities on a numerical scale from 0 (dissimilar = no species in common) to 1 (identical = all species in common). Presence-absence data have been used in similarity indices comparing fungal species composition in cysts of *H. glycines*.⁴⁵ The Bray and Curtis similarity index³¹ uses densities of each species instead of presence-absence data to calculate the similarity between two communities. To compare fungal communities of females or cysts from two sample occasions, the Bray and Curtis similarity index³¹ was used with percentage frequency data instead of density data:

$$S = \frac{2W}{A + B}$$

where S is the similarity index; A is the sum of percentage of frequency of each fungus encountered in females or cysts in one sample occasion (sampling date, stage of females or cysts, or location); B is the sum of percentages of frequency of each fungus encountered in females or cysts in another sample occasion; and W is the sum of lowest

percentages of frequency of common fungal species encountered in females or cysts in the two sample occasions. These similarity indices were used to compare compositions of the fungal communities between different stages of female and cyst development, between sampling dates, and between geographical locations. The latter comparison was calculated from data on brown cysts collected in the present study and that reported by Morgan-Jones et al.,³²⁶

Results

Overall, 2,923 (1,620 in 1991 and 1,303 in 1992) females and cysts were examined and 44% of them contained fungi. More than 35 fungal species were found, with the species composition similar in 1991 and 1992. But the fungal species and frequencies found in different stages of females and cysts were quite different (Table 3-1). Although some fungi were capable of colonizing young females, fungi were recovered from white and yellow females at low frequencies. Brown cysts were often colonized, and the frequency of colonization increased with the time of exposure to the soil (Fig. 3-1).

On each sampling date, both the number of fungal species recovered and the percentage of females or cysts colonized increased with nematode age. On 19 June 1991, when the first generation of females became mature, four species were isolated from the white and yellow females, and only 1% of 249 white females, and 3% of 118 yellow females were colonized (Table 3-1). On 3 July 1991, six species were isolated from 101 white females (13% colonized), eight species were isolated from 336 yellow females (5%

TABLE 3-1. Percentage frequency of fungal species encountered in white and yellow females and brown cysts of *Heterodera glycines* collected from a Florida soybean field during 1991 and 1992.

Fungal species	1991						1992					
	19 June			3 July			1 Oct.			20 July		
	W†	Y	W	Y	W	B	W	Y	B	W	Y	B
<i>Chaetomium cochliodes</i> Pall.	0	0	0	0	0	0.2	0	0	0	0	0	0
<i>Curvularia lunata</i> (Wakker) Boedijn	0	0	0	0	0.3	0	0	0	0	0	0	0
<i>Dicystochaeta coffeae</i> (Maggi & Persiani) Cabello & Aramburi	0	0	0	0	0	3.4	0	0	0.7	0	0	1.8
<i>Dicystochaeta heteroderae</i> (Morgan-Jones) Carris & Glawe	0	0	0	0	0	5.6	0	0	0	0	0	2.1
<i>Drechslera jagax</i> (Wall.) Shoemaker	0	0	0	0	0.3	0	0	0	0	0	0	0
<i>Exophiala pisciphila</i> McGinnis & Ajello	0	0	0	0	0.3	4.3	0	0	0.7	0.9	0	4.1
<i>Fusarium equiseti</i> (Corda) Sacc.	0	0	3.0	0	1.3	0.2	0	0	0	0	0	0.8
<i>Fusarium oxysporum</i> Schlecht.	0	0	2.0	2.4	8.3	6.6	0	0	1.0	1.8	0	4.4
<i>Fusarium semitectum</i> Berk. & Rav.	0	0	0	0.3	0	0	0	0	0	0	0	0
<i>Fusarium solani</i> (Mart.) Sacc.	0.4	0	0	0.3	5.7	11.2	0	0.9	8.4	3.5	0	11.6
<i>Fusarium</i> spp.	0	0	0	0	1.7	0.2	0	0	1.0	1.8	0	2.3
<i>Gladiolium catenulatum</i> Gilm. & Abbott	0	0	0	0	0	4.1	0	0	0	0	0	1.0
<i>Gonytrichum macrocladum</i> (Sacc.) Hughes	0.4	0	0	0	0	0	1.6	0	0	0	0	0
<i>Helicomyces</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0
<i>Hemitelia</i> sp.	0	0	1.0	0	0	0	0	0	0	0	0	0
<i>Myrothecium</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0
<i>Neocosmospora wisniefekii</i> E. F. Smith	0	0	0	0	5.3	23.3	0	0.9	10.1	4.4	0.8	18.6
<i>Paeclomyces lilacinus</i> (Thom) Samson	0	0	0	0	0	0.2	0	0	0	0	0	0.3
<i>Popilliaspora</i> sp. 1	0	0	0	0	0	0.4	0	0	0	0	0	0
<i>Popilliaspora</i> sp. 2	0	0	0	0	0	0.2	0	0	0	0	0	0
<i>Paraphoma radicina</i> (McAlp.) Morgan-Jones & White	0	0	0	0	0	0.2	0	0	0	0	0	0.5
<i>Paraphoma</i> sp.	0	0	0	0	0	0.2	0	0	0	0	0	0
<i>Periconia macrospinosia</i> Lefebvre & Johnson	0	0	0	0	0	0.2	0	0	0	0	0	0
<i>Phoma chrysanthemicola</i> Hollos	0	0	0	0	0.3	0	0	0	0	0	0	0

TABLE 3-1. (continued).

Fungal species	1992									
	1991									
	19 June		3 July		1 Oct.		20 July		9 Sept.	
	W†	Y	W	Y	B	B	W	Y	W	B
<i>Phoma medicaginis</i> Maltb. & Roum.	0	0	0	0	0.3	0	0	0	5.3	0
<i>Pseudoblastaria sojae</i> Uecker & Kulik	0	0	0	0	0	0	0	0	0.9	0
<i>Pyrenochaeta terrestris</i> (Hansen) Gorenz, Walker & Larson	0	0	0	0.3	0.3	7.6	3.2	0	6.1	0
<i>Ramichloridium subulatum</i> de Hoog	0	0	0	0	0.2	0	0	0	0	4.1
<i>Rhizoctonia solani</i> Kühn	0.4	0.9	1.0	0.3	1.3	0.6	0	0	0	0
<i>Soyadilium lingicola</i> Pesante	0	0	0	0	0.3	0	0	0	0	0.8
<i>Synonospira heteroderæ</i> Carris, Glawe & Morgan-Jones	0	0	0	0	0	5.8	0	0	0.3	0
<i>Trichoderma lignorum</i> (Tode) Harz	0	0	0	0	0	0	1.6	0	0.3	0
Sterile fungus 1 (a black yeast-like fungus)	0	2.6	0	0	9.0	10.3	6.5	0.9	13.5	17.1
Sterile fungus 2 (an extremely slow-growing fungus)	0	0	0	0	0	0	1.6	0	6.6	0
Others§	249	118	101	336	300	516	62	110	296	5.1
Total females or cysts examined	246	114	90	321	173	73	53	106	130	2.7
Number of females or cysts without fungi	98.8	96.6	89.1	95.5	57.7	14.2	85.5	96.4	43.9	0
Percentage of females or cysts without fungi										1
Number of females or cysts each colonized by more than one fungus	0	0	0	0	1	17	1	0	1	0
										22

†W=white females, Y=yellow females, B=brown cysts.

§ Fungi that would not sporulate and could not be identified.

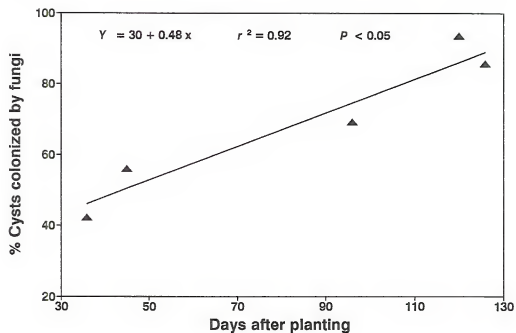


Fig. 3-1. Relationship between sampling date (x) and the percentage of brown cysts of *Heterodera glycines* with fungi (Y) in a Florida soybean field in 1991 and 1992.

colonized), and more than 20 species were isolated from 300 brown cysts (42% colonized). More than 30 species were isolated from 516 brown cysts (86% colonized) examined in the sample of 1 October 1991.

In the samples of 20 July 1992, six species were isolated from 62 white females (15% colonized), only four species were isolated from 110 yellow cysts (5% colonized), and more than 20 species were isolated from 296 brown cysts (56% colonized). In the samples of 9 September 1992, more than 11 species were isolated from 114 white females (23% colonized), only three species were isolated from 122 yellow females (3% colonized), and more than 20 species were isolated from 387 brown cysts (69% colonized). On 3 October 1992, more than 20 species were isolated from 196 of the 212 examined brown cysts (93% colonized).

The fungi isolated from white females were mainly *Rhizoctonia solani* Kühn, *Fusarium solani* (Mart.) Sacc., and *Fusarium oxysporum* Schlecht. (Table 3-1). The frequencies of *R. solani* in white and yellow females and brown cysts in samples in 1991 were similar and did not increase with time of exposure to soil. *Neocosmospora vasinfecta* E. F. Smith, *Phoma* sp., *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker & Larson, and a black yeast-like fungus were also encountered at a relatively high frequency in white females in the samples collected in 1992, but not in 1991. The fungi most frequently encountered in yellow females were *Fusarium* spp., *R. solani*, *N. vasinfecta*, and the black yeast-like fungus. The common species encountered in brown cysts were *N. vasinfecta*, *F. oxysporum*, *F. solani*, *Dictyochaeta heteroderae* (Morgan-Jones) Carris & Glawe, *D. coffeae* (Maggi & Persiani) Cabello & Arambarri, *Exophiala*

pisciphila McGinnis & Ajello, *Gliocladium catenulatum* Gilm. & Abbott, *P. terrestris*, and *Stagonospora heteroderae* Carris, Glawe & Morgan-Jones. *Dictyochaeta heteroderae*, *D. coffeae*, *G. catenulatum*, and *S. heteroderae* were isolated only from brown cysts. Numerous other fungi were isolated from brown cysts at low frequencies (Table 3-1).

The similarity indices between sampling dates for the fungi in white females, yellow females, or brown cysts are listed (Table 3-2). The average of similarity indices of the fungi in brown cysts at different sampling occasions was 0.54, with a standard deviation (SD) of 0.12. In contrast, the averages of the similarity indices for fungi in white or yellow females versus white females, yellow females, or brown cysts were low (Table 3-2). The average of the similarity indices for fungi in brown cysts in our study compared with the fungi of brown cysts from other locations surveyed by Morgan-Jones et al.³²⁶ was 0.4, whereas the average in brown cysts among the soils collected by Morgan-Jones et al. was 0.66 (Table 3-3).

Discussion

The fungi isolated from the females and cysts by the methods used in our study and others^{45,157,158,311,326,328,334} are typically opportunists. Obligate parasites of females or eggs of the nematodes generally are not isolated by these methods. The species of *Cylindrocarpon*, *Exophiala*, *Fusarium*, *Gliocladium*, *Paecilomyces*, *Phoma*, and *Verticillium* were among the fungi most frequently encountered from nematode cysts in other studies.^{331,332} These species appear to colonize eggs of a range of plant-

TABLE 3-2. Similarity indices between sampling dates for fungi in white and yellow females and brown cysts of *Heterodera glycines* collected from a Florida soybean field during 1991 and 1992.

	White females				Yellow females				Brown cysts			
	1991		1992		1991		1992		1991		1992	
	3 July	20 July	9 Sept.		19 June	3 July	20 July	9 Sept.	3 July	1 Oct.	20 July	9 Sept.
White females												
19 June, 1991				(0.03 ± 0.04; n = 6) ^f				(0.12 ± 0.09; n = 16)				(0.17 ± 0.12; n = 20)
3 July, 1991		0	0.03	0.07	0	0.17	0.21	0.17	0.04	0.02	0.03	0.02
20 July, 1992			0.11		0.12	0.30	0	0.12	0.16	0.06	0.06	0.08
9 Sept., 1992			0.03		0	0	0.09	0.09	0.24	0.19	0.33	0.25
					0.19	0.15	0.20	0.12	0.44	0.24	0.33	0.31
												0.22
Yellow females												
19 June, 1991				(0.26 ± 0.20; n = 6)								(0.08 ± 0.03; n = 20)
3 July, 1991				0.08	0.25	0.54			0.15	0.07	0.11	0.09
20 July, 1992					0.08	0.09			0.14	0.07	0.06	0.09
9 Sept., 1992						0.53			0.12	0.06	0.09	0.07
									0.11	0.05	0.05	0.06
												0.04
Brown cysts												
3 July, 1991												(0.54 ± 0.12; n = 10)
1 Oct., 1991									0.43	0.40	0.48	0.42
20 July, 1992										0.53	0.73	0.74
9 Sept., 1992											0.63	0.43
												0.61

The similarity index: $S = 2W / A + B$, where A is the sum of percentage of frequency of each fungus encountered in females or cysts in one sample occasion, B is the sum of percentage of frequency of each fungus encountered in females or cysts in another sample occasion, and W is the sum of lowest percentages of frequency of common fungal species encountered in females or cysts in the two sample occasions. The values in parentheses are averages of similarity indices ± standard deviations for the group of data directly beneath.

TABLE 3-3. Similarity indices between locations and sampling dates for fungi in brown cysts of *Heterodera glycines* collected in different locations in the southeastern United States at different times.

	Mid-brown cysts from dried soil in 1981†				Brown cysts from a Florida field soil			
	Mississippi		Alabama		1991		1992	
					3 July	1 Oct.	20 July	9 Sept. 3 Oct.
1981								
Florida				(0.66 ± 0.05; n = 6)‡	(0.41 ± 0.08; n = 8)		(0.40 ± 0.10; n = 12)	
Mississippi	0.64	0.56	0.63		0.31	0.39	0.21	0.31
Alabama		0.71	0.68		0.31	0.39	0.27	0.38
Missouri			0.72		0.35	0.49	0.32	0.44
					0.49	0.51	0.37	0.47
								0.52
1991								
3 July, 1991							(0.55 ± 0.14; n = 6)	
1 Oct., 1991							0.40	0.48
							0.53	0.73
								0.74
1992								
20 July, 1992							(0.56 ± 0.10; n = 3)	
9 Sept., 1992							0.63	0.43
								0.61

† Data cited from Morgan-Jones et al. (1981).

‡ The values in parentheses are averages of similarity indices ± standard deviations for the group of data directly beneath.

parasitic nematodes.³³² In addition, species of *Stagonospora* and *Paraphoma* also have been isolated frequently from cysts of *H. glycines*.⁴⁵ The most common species isolated in our study, except for *N. vasinfecta* and the black yeast-like fungus, also were in these groups of fungi. *Pyrenochaeta terrestris* was probably identified as *Phoma terrestris* Mont. in some studies.¹⁵⁸ *Dictyochoaeta heteroderæ* (as *Codinaea heteroderæ* Morgan-Jones) was reported with a relative high frequency from a Florida soil.³²⁶ *Neocosmospora vasinfecta* was the most frequently occurring species from brown cysts in our study. This fungus is probably adapted to the high temperature of a tropical or subtropical climate,¹⁰⁸ but its impact on eggs in cysts of SCN is unclear.

The frequencies of fungi in young females were much lower in our field study than in those reported in previous greenhouse studies.^{157,158} It is possible that more favorable conditions existed in the greenhouse. The frequency of fungi in yellow females was lower than that in white females (Table 3-1). This suggests that resistance to fungal infections may increase as females change from white to yellow.

The similarity indices between fungi in brown cysts in our study and fungi of brown cysts reported by Morgan-Jones et al.³²⁶ were much lower (0.4) than indices for fungi in brown cysts among soil samples from various locations in the study of Morgan-Jones et al. (0.66), even though one soil sample in that study was also from Florida. This suggests that the fungi are influenced greatly by environmental conditions or by methodology. Morgan-Jones et al.³²⁶ extracted cysts from air-dried soils stored for a period of time, rather than processing these soils immediately after collection, as was done in this study.

Under field conditions, no significant difference was observed between the group of similarity indices that were calculated for fungi in brown cysts from two sampling dates within a year (1991 vs. 1991, 1992 vs. 1992) and the group of similarity indices that were calculated from two dates in different years (1991 vs. 1992) (Table 3-2). This suggested that the environmental conditions in the two growing seasons were similar. The similarity index for fungi in brown cysts between two sampling dates, however, was negatively correlated with the length of time between the dates without considering the year (Fig. 3-2). The fungi may have been affected by either the seasonal changes in field conditions, including the climate and cultural conditions, or the condition of cysts in soil, or both. Between the dates 1 October 1991 and 3 October 1992, for example, the length of time was considered to be 2 days rather than 2 days plus 1 year; the environmental conditions, the condition of cysts, and the exposure periods of the cysts to the soil were similar and, therefore, the similarity index was as high as 0.74 (Table 3-2). In contrast, between the dates 3 July and 1 October in the same year of 1991, the length of time was 90 days; the environmental conditions, the condition of cysts, and the exposure periods of cysts to the soil were much different; and the similarity index was as low as 0.43. The similarity indices for fungi in white or yellow females versus white females, yellow females, or brown cysts was low because no one single fungal species predominated and none infected the females consistently.

Although several species occurred more frequently than others, no single species predominated in white and yellow females and in brown cysts. Our study and previous work^{45,158,330} indicate that cysts colonized by one fungus are not readily colonized

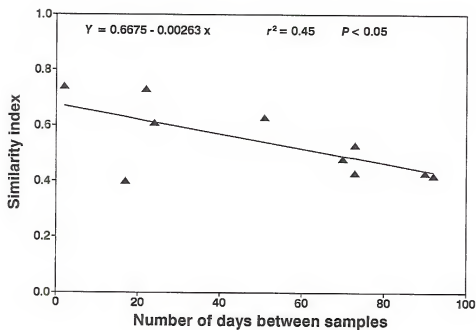


Fig. 3-2. Relationship between the length of time between two sampling dates (x) and the similarity index (Y) for fungi in brown cysts of *Heterodera glycines* between those two dates in a Florida soybean field in 1991 and 1992. The days between sampling dates were calculated without considering the years; for example, the time between 1 October of 1991 and 3 October of 1992 is 2 days rather than a year plus 2 days; the corresponding similarity index associated with these two sampling dates is 0.74 (see Table 3-2).

by other fungi. Only two of 525 white females, none of the yellow females, and 66 of 1,711 brown cysts were colonized by more than one fungus (Table 3-1). Because of this and because these opportunistic fungi are affected greatly by the environmental conditions, it may be important to create an environment favoring the development of nematode-pathogenic fungi in order to stimulate rapid colonization of cysts of SCN. For example, addition of crustacean chitin to soil improved the control of SCN.³⁹⁵ The impact of soil amendments on manipulating the soil mycoflora to control SCN needs further investigation.

CHAPTER 4
POPULATION DEVELOPMENT OF *HETERODERA GLYCINES*
IN RESPONSE TO MYCOFLORA IN SOIL FROM FLORIDA

Introduction

Environmental concern about the use of pesticides in recent years has stimulated interest in biological control of nematodes.⁴⁴⁸ Searching for nematophagous fungi is an important aspect of biological control of nematodes. Before the 1970's, reports on predaceous and endoparasitic fungi that attack vermiform nematodes dominated the literature on biological control of nematodes. Although fungi colonizing females and cysts of sedentary endoparasitic nematodes have been known for over a century, not until recent years has research on these fungi increased.⁴⁴⁷ Natural controls by these fungi have been reported from several countries and on various nematodes (Chapter 1).

Although many species of fungi have been isolated from cysts of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe (Chapter 1, Table 1-2), the impact of these fungi on the nematode population in soil is poorly understood. In the present study, the species and frequencies of fungi colonizing females and cysts of SCN in a Florida soybean field were elucidated (Chapters 2 and 3). The common fungi isolated from the females and cysts in this field were *Neocosmospora vasinfecta* E. F. Smith, *Fusarium solani* (Mart.) Sacc., *Fusarium oxysporum* Schlecht., *Dictyochaeta coffeae* (Maggi & Persiani) Cabello & Arambarri, *Dictyochaeta heteroderae* (Morgan-Jones)

Carris & Glawe, *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker & Larson, *Exophiala pisciphila* McGinnis & Ajello, *Gliocladium catenulatum* Gilm. & Abbott., *Stagonospora heteroderae* Carries, Glawe & Morgan-Jones, and a black, yeast-like fungus (Chapter 3).

The objective of this study was to evaluate the effects of mycoflora in the soil from the soybean field on the population development of *H. glycines* in a greenhouse bioassay. Several methods have been used for detecting suppression of nematodes by soil-borne fungi (Chapter 1). In the present study, different soil treatments were used in four experiments to create mycoflora that were different from that in untreated field soil.

Materials and Methods

Effects of mycoflora on the nematode population in untreated soil or soil treated by autoclaving or microwave heating (Experiment 1). Soil was collected on 30 May 1992 with a bucket auger (10 cm in diameter) from six plots in a soybean field at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida. The soil texture was 90.5% sand, 5.3% silt, 4.2% clay, with 1.6% organic matter and pH 5.7. The density of SCN was one cyst and one second-stage juvenile (J2)/100 cm³ of soil. The soil was passed through a 6-mm-aperture sieve, mixed, and either treated with microwave heating for 4 or 6 minutes, autoclaved at 120 C for 20 minutes, or left untreated. For microwave heating treatments,¹³⁷ 1-kg lots of soil were placed in even layers in open plastic bags and heated in a microwave oven (650 Watts) for the time required. The soil was then placed in pots 15 cm high × 15 cm in diameter and infested

with SCN or used untreated. Thus, the experiment was a factorial design with four soil treatments, two nematode levels, and 10 replicates.

The nematodes added into the soil were yellow females and light brown cysts of *H. glycines* race 3 obtained from roots of 6-week-old soybean plants growing in a greenhouse. The females and cysts washed from the soybean roots with a vigorously applied water stream through a 600- μ m-opening sieve onto a 250- μ m-opening sieve and extracted by modified centrifugal flotation.²²⁵ The nematode with debris were suspended in a solution of 0.4 kg of sucrose/liter of water and centrifuged for 5 minutes at about 7,200 g, and the supernatant was discarded. The contents in the tube were suspended again in a solution of 0.5 kg of sucrose/liter of water and centrifuged again for 5 minutes. The supernatant that contained uniform and clean yellow females and light brown cysts was collected and used as inoculum.

The nematodes were thoroughly mixed into soil on 21 June at 707 ± 47 (0.1 cm^3) females and cysts per pot. The pots were placed in a greenhouse and covered with aluminum foil to reduce fungal recolonization before planting soybean seeds. The pots were planted twice to soybean cv. Cobb. Soybean seeds were treated with 0.5% NaOCl for 3 minutes and coated with Nitragin powder, a nitrogen-fixing bacterial inoculum (*Rhizobium* spp.), at the rate of 1:450 (w/w of the inoculum/seeds). The first planting was done at week 2 (2 weeks after adding the nematode into soil; all of the following time schedules were based on weeks after adding nematodes into soil); seven seeds were added to each pot and after 1 week plants were thinned to provide five plants in each pot. Forty-six days after planting, the shoots of soybean plants were removed, and the plant

heights and fresh shoot weights were recorded. Two days after removing the soybean plants, seven soybean seeds were planted in each pot of soil containing the remaining root system, and after 1 week plants were thinned to provide three plants in each pot. The soybean plants were removed 51 days after planting, and the fresh shoot weights and heights were recorded.

During the soybean growing period, the pots were watered and esfenvalerate, hexakis, and an insecticidal soap were used to control insects or mites when needed. The lowest and highest daily temperatures (mean $\pm$ SD) in the greenhouse were 21.6 ± 2.0 and 38.8 ± 0.8 , 22.4 ± 0.6 and 40.6 ± 1.3 , 21.1 ± 1.8 and 37.3 ± 3.4 , and 17 ± 0.9 and 36.6 ± 0.9 , in July, August, September, and early October, respectively.

At weeks 2 and 6, about 50 g of soil were taken from each pot containing nematodes and J2 were extracted by using a modified Baermann funnel technique³⁸² with an incubation period of 40 hours. The numbers of J2 were recorded. At the second harvesting date (week 16), 70-100 g of soil were taken from each of the nematode-infested pots. Cysts and J2 were extracted from the soil samples by modified centrifugal flotation.²²⁵ The soil was washed into a cup, and after 30 seconds the soil suspension was decanted onto a set of nested sieves with 600-, 250-, and 40- μ m apertures. The contents on the 40- μ m-aperture sieve were collected for extraction of J2. The soil in the cup was washed three times and the supernatant was decanted immediately onto the 600- and 250- μ m-aperture sieves. The cysts with soil debris remaining on the 250- μ m-aperture sieve were collected. The cysts were separated from the soil and inorganic debris by centrifugation in 1.5 kg of sucrose/liter of water. The numbers of J2 and cysts

were recorded. The number of eggs was calculated from the number of cyst per pot multiplied by the number of eggs per cyst that was recorded during the observation of fungal parasitism at week 31. After the second harvest of soybean plants, the pots were kept for post-harvest incubation and watered every 2-3 days to maintain the soil moisture. At week 29, the J2 were extracted from the nematode-infested soil (40-70 g of soil per pot) by centrifugal flotation.²²⁵ At week 31, cysts were extracted from five pots of each nematode-infested soil for determining fungal colonization. Cysts were randomly picked, placed on slides, covered with a cover glass, ruptured by pressing the cover glass, and observed under a light microscope. Ten cysts were examined from each treatment. The percentage of cysts colonized and percentage of eggs parasitized by fungi were recorded. Because *Arthrobotrys dactyloides* Drechsler was found frequently in cysts in soil treated with microwave heating for 4 or 6 minutes and in autoclaved soil, the percentages of cysts colonized by this fungus, based on the existence of constricting rings in cysts, were also recorded. The index of fungal parasitism of eggs was recorded on a scale from 0-10: 0 = no eggs parasitized, 1 = 1-10% eggs parasitized, 2 = 11-20% eggs parasitized, up to 10 = 91-100% eggs parasitized.

Effects of dilution of fungal density on the nematode population (Experiment 2).

This experiment included five soil treatments and five replicates. The soil used in this experiment was the same batch of soil as that used in experiment 1. The soil was treated with microwave heating for 4 minutes, and mixed with untreated soil at a ratio of 1:0 (0% untreated soil), 3:1 (25% untreated soil), 1:1 (50% untreated soil), 1:3 (75% untreated soil), and 0:1 (100% untreated soil). All other procedures and measurements

were the same as in experiment 1, except that the last measurements of J2 densities were done at week 31 instead of week 29 as in experiment 1.

Effects of mycoflora on nematode population in soil stored at room temperature compared to soil treated with formalin (Experiment 3). This experiment was a 2 (soil treatments) $\times$ 2 (nematode levels) factorial design with 10 replicates. The soil used in this experiment was the same batch of soil as used in experiments 1 and 2. The soil was either treated with formalin or stored at room temperature. For the formalin treatment, the soil was treated with 40% formaldehyde at a rate of 3.8 ml/liter of soil^{78,245}, sealed in a plastic tray covered with plastic wrap, and maintained for 3 days in a greenhouse. The soil was placed in pots 15 cm high and 15 cm in diameter, and leached for 11 days by drenching with water. The nematodes were added into soil in the same way as in experiment 1. Soil stored at room temperature without addition of nematodes was also included for a background control. The pots were covered with aluminum foil for 2 weeks. Following the treatment, soybean seeds were planted with the same procedures used for the first planting in experiment 1. At week 6, the soybean plant shoots were removed, and fresh shoot weights and heights were recorded. The J2 densities were determined for 50-120 g of soil collected from each pot at weeks 2, 6, 11, 19, and 27. The J2 were extracted with the modified Baermann funnel technique,³⁸² except that the J2 in samples taken in week 27 were extracted by centrifugal flotation.²²⁵ Fungal colonizations were determined from the sample taken at week 27 with the same methods as described in experiment 1.

Effects of mycoflora on nematode population in five soils treated with microwave heating or left untreated (Experiment 4). This was a 5 (soils) $\times$ 2 (soil treatments) $\times$ 2 (nematode levels) factorial design with six replicates. Soil samples were taken on 24 February 1993 from five field sites: i) a soybean field (soil 1) at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida; ii) a soybean breeding field (soil 2) on campus of the University of Florida; iii and iv) a peanut breeding field (soil 3) and a fallowed field (soil 4) at the same farm as soil 1; and v) a fallowed field (soil 5) near Williston, Levy County, Florida. Each soil was passed through a 1-cm-aperture sieve, mixed, and either used untreated or treated with microwave heating for 4 minutes as described above. The treated and untreated field soils were placed in pots 10 cm high $\times$ 10 cm in diameter in a greenhouse. The texture of untreated soil and chemical characteristics of both treated and untreated soils were determined and summarized in Table 4-1.

Soybean seeds were treated with 0.5% NaOCl for 3 minutes, placed on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) in petri dishes, and incubated at 28 C in a growth chamber for 3 days. Germinated seeds without signs of fungal growth were selected. One seed was placed in each pot. After 7 days the plants were inoculated with J2 obtained by a method modified from a technique described by Lauritis et al.²⁷² The cysts were extracted from roots of soybean cultured in the greenhouse. All solutions and equipment used were sterilized, and the procedures were conducted under sterile conditions. The extracted cysts were washed with sterile water, and put on six layers of tissue on a screen placed in a jar containing a solution of 4 mM ZnCl₂ plus 100 mg of

TABLE 4-1. Texture and chemical characteristics of soil collected from five sites in Florida.

Soil source	% clay	% silt	% sand	% o.m.	pH	N	P	K	Ca	Mg
Untreated										
Soybean field, Green Acres	4.2	5.3	90.5	1.63	5.7	1.5	52	24	196	37
Soybean field, Campus	5.2	3.8	91.0	2.19	5.7	1.3	131	51	307	25
Peanut field, Green Acres	4.3	4.7	91.0	1.94	5.3	1.5	54	19	170	9
Fallow field, Green Acres	4.2	4.1	91.7	1.53	5.7	1.5	52	8	222	20
Fallow field, Williston	5.0	5.5	89.1	1.44	7.5	3.0	21	89	1,030	39
Mean	4.6	4.7	90.7	1.77	6.0	2.0	62	38	385	26
Treated with microwave heating for 4 minutes										
Soybean field, Green Acres					6.0	1.0	55	28	219	39
Soybean field, Campus					5.8	3.5	89	36	205	17
Peanut field, Green Acres					6.3	2.0	40	52	562	23
Fallow field, Green Acres					5.7	1.5	48	8	223	20
Fallow field, Williston					7.8	3.0	22	90	1,150	39
Mean					6.3	2.2	51	43	472	28

The type of all five soils is Arredondo fine sand. Soil elements were reported as mg/kg of soil. No differences in element level or pH of soil were observed between soil treatments. O.m. = organic matter.

streptomycin, 50 mg of chlortetracycline, and 20 mg of quinolinol/liter of water. The J2 hatched during the first day were discarded, the solution was replaced, and the contents were incubated again for 2 days. The newly hatched J2 were transferred to another six layers of tissue paper with a freshly prepared solution containing 100 mg of streptomycin, 50 mg of chlortetracycline and 20 mg of quinolinol/liter of water. The second batch of J2 was washed with sterile water, rinsed with 0.001% HgCl_2 for 5 minutes, and rinsed with sterile water on a 10- μm -aperture nylon filter mounted at one end of a tube. No fungal growth was observed after plating the nematode suspension on PDA for 3 days at 23-24 C. The J2 suspension was adjusted to 400 J2/ml. On 4 March, three holes about 3 cm deep and 1 cm in diameter were made around the soybean plants, and 2,000 J2 (5 ml of nematode suspension) were added to each pot.

The pots were maintained in a greenhouse and watered daily. Esfenvalerate, hexakis, and an insecticidal soap were used to control insects or mites when needed. The lowest and highest daily temperatures (mean $\pm$ SD) in the greenhouse were 11.7 ± 5.5 and 33.1 ± 4.2 , 17.1 ± 2.8 and 34.7 ± 1.2 , 19.8 ± 0.5 and 36.0 ± 1.4 , and 20.0 ± 0 and 38.4 ± 0.7 C, in March, April, May and early June, respectively.

Fourteen weeks after inoculation of the nematode, the plants were removed, and the fresh shoot weights and heights were recorded. Soil and root samples from about half of each pot were removed and weighed. Second-stage juveniles and cysts were extracted by the same procedure described in experiment 1, except that the roots were scrubbed to release the females and cysts. The densities of J2 and cysts were recorded. The percentage of cysts colonized, percentage of eggs parasitized by fungi, and index of

parasitism of eggs were recorded by procedure described above. The cysts from each pot were then collected and the eggs were released by grinding with a glass tissue grinder. Egg suspensions were prepared and the egg densities were recorded. After recording the egg densities, all eggs in each untreated soil or all eggs from all soil treated with microwave heating were mixed. The eggs were washed with sterile water on a 25- μ m-aperture sieve. The density of eggs in each suspension was adjusted to 30 eggs/ml. One ml of the egg suspension was added to each petri dish containing water agar plus 100 mg of streptomycin, and 50 mg of chlortetracycline/liter of medium; 10 dishes were used for each egg sample. The fungi growing from eggs were transferred and identified by following the procedures described previously in Chapter 2.

The nematode densities were transformed to $\log_{10}(x + 1)$ values and the percentages of fungal colonization of eggs and cysts were transformed to arcsin (x) values. All data were subjected to analysis of variance (ANOVA). The means were compared with Duncan's multiple-range test. Linear regressions were performed to determine the relationship between the nematode density and fungal parasitism of eggs.

Results

Effects of mycoflora on the nematode population in untreated soil or soil treated by autoclaving or microwave heating. The densities of J2 were lower in autoclaved soil or soil treated with microwave heating for 4 or 6 minutes than in untreated soil at weeks 2 and 29, but no differences were observed in the J2 density among soil treatments at

weeks 6 and 16 (Table 4-2). The densities of cysts, which were evaluated at week 16 were lower in autoclaved soil or soil treated with microwave heating for 4 or 6 minutes than that in untreated soil. The nematode population in untreated soil without addition of nematodes increased from the initial field density of one cyst and 1 J2 to 231 cysts and 314 J2/100 g of soil at week 16, and 434 J2/100 g of soil at week 29. No difference in nematode density was detected between untreated soil with nematodes added and untreated soil without addition of nematodes. No difference in percentage of cysts colonized by fungi was detected among soil treatments (Table 4-2). No correlation was observed between nematode density and percentage of cysts colonized by fungi. However, a high frequency of colonization of cysts by *A. dactyloides* was encountered in the soil treated with microwave heating for 4 or 6 minutes or in autoclaved soil and the nematode density was negatively correlated with percentage of cysts colonized by *A. dactyloides*.

Effects of dilution of fungal density on the nematode population. The J2 density in soil containing 0% untreated soil at week 31 was lower than that in all other treatments (Table 4-3). At week 16, the J2 density was higher in soil containing 25% untreated soil than in soil containing 0% or 75% untreated soil. Otherwise, no other difference in nematode density was observed among soil fractions (Table 4-3). *Arthrobotrys dactyloides* was encountered at a high frequency in the cysts in soil containing 0% untreated but not found in soil containing 25-100% untreated soil. No correlation was observed between nematode density and percentage of cysts colonized by fungi, whereas the nematode density was correlated with the density of *A. dactyloides*.

TABLE 4-2. Nematode densities and fungal colonization of cysts and eggs of the soybean cyst nematode, *Heterodera glycines*, in soil autoclaved (Aut), treated with microwave heating for 4 (MW4) or 6 (MW6) minutes, or untreated (Unt).

Soil treatment	Second-stage juveniles /100 g of soil					Cysts/ 100 g soil	Eggs/ g soil	% Cysts colonized by fungi	% Cysts colonized by <i>Arthrobotrys</i> <i>dactyloides</i>	% Eggs parasitized by fungi	Index of fungal parasitism of eggs
	Week 2	Week 6	Week 16	Week 29							
Aut + nematode	200 c	71 a	387 a	84 b	169 bc	122 b	89 a	23 b	50 a	5.1 a	
MW6 + nematode	294 bc	97 a	298 a	37 c	158 bc	95 b	92 a	43 a	36 a	4.0 a	
MW4 + nematode	426 b	94 a	213 a	55 bc	103 c	86 b	86 a	13 b	28 a	2.8 a	
Unt + nematode	788 a	155 a	523 a	244 a	275 a	140 ab	88 a	0 c	18 a	2.4 a	
Unt			314 a	433 a	231 ab	286 a	92 a	0 c	19 a	2.3 a	

The data are means of 10 replicates except the second-stage juveniles/g soil at week 29, eggs/g soil, % eggs parasitized by fungi, and index of fungal parasitism of eggs, which are means of five replicates. The nematode population densities were transformed to $\log_{10}(x + 1)$ values and the percentage data were transformed to arcsin (x) values before being subjected to ANOVA. The same letter in columns indicates no significant differences at $P \leq 0.05$ according to Duncan's multiple-range test. Index of fungal parasitism of eggs: 0 = no eggs parasitized, 1 = 1-10% of the eggs parasitized, 2 = 11-20% of the eggs parasitized, up to 10 = 91-100% of the eggs parasitized by fungi.

TABLE 4-3. Nematode densities and fungal colonization of cysts and eggs of the soybean cyst nematode, *Heterodera glycines*, in untreated soil mixed with soil treated with microwave heating for 4 minutes.

Soil treatment	Second-stage juveniles /100 g of soil					Cysts/ 100 g soil	Eggs/ g soil	% Cysts colonized by fungi	% Cysts colonized by <i>Arthrobotrys</i> <i>dactyloides</i>	% Eggs parasitized by fungi	Index of fungal parasitism of eggs
	Week 2	Week 6	Week 16	Week 31							
0% untreated soil	536 a	88 a	175 b	38 b	71 a	86 a	13 a	28 a		2.8 a	
25% untreated soil	409 a	230 a	484 a	147 a	114 a	160 a	82 a	0 b	22 a	3.1 a	
50% untreated soil	566 a	167 a	254 ab	132 a	113 a	165 a	90 a	0 b	29 a	3.6 a	
75% untreated soil	685 a	52 a	175 b	178 a	100 a	131 a	82 a	0 b	34 a	3.8 a	
100% untreated soil	787 a	131 a	279 ab	152 a	126 a	140 a	88 a	0 b	18 a	2.4 a	

The data are means of five replicates. The nematode population densities were transformed to $\log_{10}(x + 1)$ values and the percentage data were transformed to arcsin (x) values before being subjected to ANOVA. The same letter in columns indicate no significant differences at $P \leq 0.05$ according to Duncan's multiple-range test. Index of fungal parasitism of eggs: 0 = no eggs parasitized, 1 = 1-10% of the eggs parasitized, 2 = 11-20% of the eggs parasitized, up to 10 = 91-100% of the eggs parasitized by fungi.

Effects of mycoflora on nematode population in soil stored at room temperature compared to soil treated with formalin. A higher J2 density was observed in formalin-treated soil than in untreated soil at week 6 (Table 4-4). No differences in J2 density at other sample occasions or differences in densities of cysts and eggs were observed between formalin-treated and untreated soil into which nematodes were added. Although the nematode density in untreated soil, into which no nematodes were added, increased from an original density of 1 J2/100 cm³ of soil to 19, and 31 J2/100 cm³ of soil at weeks 19, and 27, respectively, it was still lower than that in either untreated or formalin-treated soil into which nematodes were added. Although formalin treatment suppressed fungal colonization of eggs (Table 4-4), no correlation was observed between nematode density and percentage of cysts colonized by fungi.

Effects of mycoflora on nematode population in five soils treated with microwave heating or left untreated. The nematode densities in soil treated with microwave heating were 4.3 fold higher for cysts, 7.1 fold higher for eggs (Table 4-5), and 7.5-fold higher for J2, in comparison with untreated soil (Table 4-5). However, no difference in nematode density was observed among soil sources. The number of eggs produced per female was 73% higher in soil treated with microwave heating than in untreated soil ($P \leq 0.05$, Table 4-5). An average of 98% cysts was colonized by fungi in untreated soil, compared to only 47% cysts colonized in soil treated with microwave heating. Egg density was negatively correlated either with percentage of cysts colonized by fungi or the index of fungal parasitism of eggs in experiment 4 (Fig. 4-1). A similar correlation was observed using J2 or cyst density instead of egg density. Therefore, it was

TABLE 4-4. Nematode densities and fungal colonization of cysts and eggs of the soybean cyst nematode, *Heterodera glycines*, in untreated soil or soil treated with formalin.

Soil treatment	Second-stage juveniles /100 g of soil at weeks					Cysts/ 100 g soil	Eggs/ 100 g soil	% Cysts colonized by fungi	% Cysts colonized by <i>Arthrobotrys</i> <i>dactyloides</i>	% Eggs parasitized by fungi	Index of fungal parasitism of eggs
	2	6	11	19	27						
Formalin + nematode	588 a	261 a	682 a	95 a	71 ab	146 a	267 a	78 a	2 a	4 b	0.2 b
Untreated + nematode	539 a	106 b	579 b	84 a	147 a	106 a	196 a	86 a	0 a	13 a	1.3 a
Untreated				19 b	31 b						

The data are means of 10 replicates except the second-stage juveniles/100 g soil at week 27, eggs/g soil, % eggs parasitized by fungi, and index of fungal parasitism of eggs, which are means of five replicates. The nematode population densities were transformed to $\log_{10}(x + 1)$ values and the percentage data were transformed to arcsin (x) values before being subjected to ANOVA. The same letter in columns indicate no significant differences at $P \leq 0.05$ according to Duncan's multiple-range test. Index of fungal parasitism of eggs: 0 = no eggs parasitized, 1 = 1-10% of the eggs parasitized, 2 = 11-20% of the eggs parasitized, up to 10 = 91-100% of the eggs parasitized by fungi.

TABLE 4.5. Densities and fungal colonization of cysts and eggs of the soybean cyst nematode, *Heterodera glycines*, in soil collected from five sites and either treated with microwave heating for 4 minutes or left untreated.

Soil sources	12/ 100 g soil	Cysts/ 100 g soil	Eggs/ g soil	% Cysts colonized by fungi	% Eggs parasitized by fungi	Index of fungal parasitism of eggs
Untreated soil						
Soybean field, Green Acres	24	174	63	95	64	6.2
Soybean field, Campus	10	131	43	97	57	5.5
Peanut field, Green Acres	27	281	79	97	73	7.1
Fallow field, Green Acres	790	381	218	100	68	7.1
Fallow field, Williston	7	214	94	100	78	7.9
Mean	172	236	99	98	66	7.1
Soil treated with microwave heating						
Soybean field, Green Acres	251	552	429	48	23	2.3
Soybean field, Campus	483	753	483	38	9	1.0
Peanut field, Green Acres	1,158	1,130	867	43	12	1.2
Fallow field, Green Acres	233	920	490	38	24	2.1
Fallow field, Williston	1,330	1,752	1,219	69	33	3.3
Mean	691	1,022	698	47	20	2.0

The data are means of six replicates. The nematode population densities were transformed with $\log_{10}(x + 1)$ and the percentage data were transformed with arcsin (x) before being subjected to ANOVA. The averages of measurements of the five soils are higher for nematode densities and lower for fungal colonization and index of parasitism of eggs in treated soil than in untreated soil according to ANOVA ($P \leq 0.01$). No significant differences in nematode population densities, percentages of cysts colonized by fungi, fungal parasitism of eggs among soils, and no interaction between soil source and soil treatment, were observed. Index of fungal parasitism of eggs: 0 = no eggs parasitized, 1 = 1-10% of the eggs parasitized, 2 = 11-20% of the eggs parasitized, up to 10 = 91-100% of the eggs parasitized by fungi.

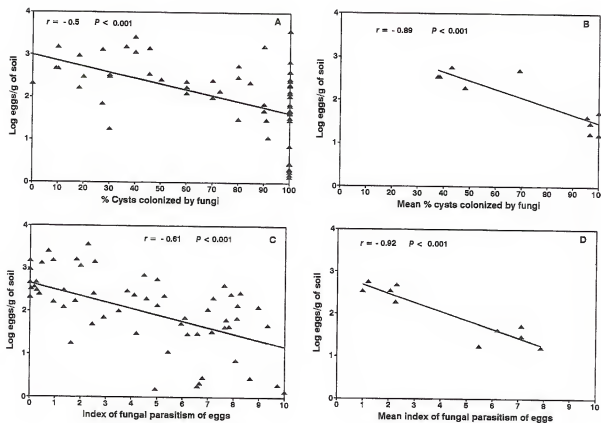


Fig. 4-1. Relationship between the nematode population and fungal colonization of cysts or parasitism of eggs. Data were obtained in experiment 4. A and B) Linear correlation between log eggs/g soil and percentage of cysts colonized by fungi; C and D) linear correlation between log eggs/g soil and index of fungal parasitism of eggs. A and C) measurements from each replicate; B and D) mean measurements of six replicates of each treatment. Index of fungal parasitism of eggs: 0 = no eggs parasitized, 1 = 1-10% of the eggs parasitized, 2 = 11-20% of the eggs parasitized, up to 10 = 91-100% of the eggs parasitized by fungi.

concluded that the low nematode densities in the untreated soil in experiment 4 were attributed to fungal colonization of the nematode cysts and eggs.

A total of over 16 species of fungi were isolated from eggs in the soil from the five sites (Table 4-6). A black, yeast-like fungus (unidentified sp. 1) was recovered at a high frequency in all five soils. *Fusarium oxysporum* and a species of *Humicola* were also common in the five soils. Another unidentified sterile species (sp. 2) was recovered at a high frequency in soil 1. *Paraphoma radicina* (McAlp.) Morgan-Jones & White was recovered in soil 3 at a high frequency. In soil treated with microwave heating, only a few fungi were found in eggs and these were at low frequencies.

Effects of soil treatments and nematodes on plant growth. In experiments 1 and 3, SCN at an initial density of about 50 females and cysts/100 g of soil generally suppressed soybean plant growth in the first planting (Table 4-7). When nematodes were added, the growth of soybean plants in autoclaved soil, or in soil treated with microwave heating for 4 or 6 minutes or with formalin, was generally greater than that in untreated soil. In experiment 2, the soybean plant growth was better in soil containing 0% untreated soil than in soil containing 25-100% untreated soil (Table 4-7). In experiment 4, plant growth varied among soil sources, but there was an interaction between soil treatment and soil sources (Table 4-7).

Discussion

Population densities of SCN were affected by fungi that colonize cysts and eggs, but the effects varied among four experiments. The untreated soil showed little

TABLE 4-6. Species and frequency of fungi isolated from eggs of *Heterodera glycines* in soil collected from five sites in Florida.

Fungal species	Number of eggs encumbered with fungi (total 300 eggs examined)					Soil treated with MW4 †
	Untreated soil					
	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	
<i>Fusarium oxysporum</i> Schlecht.	1	10	4	2	7	3
<i>Fusarium solani</i> (Mart.) Sacc.	1	0	0	0	0	0
<i>Humicola</i> sp. 1	3	3	8	8	2	0
<i>Humicola</i> sp. 2	0	0	1	0	0	0
<i>Microspheeropsis olivacea</i> (Bonord.) Hoehnelt	0	0	1	0	0	0
<i>Neocosmospora vasinfecta</i> E. F. Smith	1	0	0	0	0	0
<i>Puccinomyces inflatus</i> (Burnside) Carmichael	0	0	0	0	1	0
<i>Paraphoma radicina</i> (McAlp.) Morgan-Jones & White	0	1	30	8	4	0
<i>Phoma</i> sp.	0	8	0	0	0	0
<i>Pyrenochaeta terrestris</i> (Hansen) Gorenz, Walker & Larson	1	1	0	2	0	0
<i>Rhizoctonia solani</i> Kühn	1	0	0	1	0	0
<i>Sclerobasidium terreum</i> Abbott	0	1	0	0	0	0
<i>Sclerobasidium tricladium</i> Matsushima	0	0	0	0	0	1
<i>Stagonospora heteroderæ</i> Carris, Glawe & Morgan-Jones	0	1	0	0	0	0
Unidentified sp. 1 (black yeast-like fungus)	12	39	28	18	9	0
Unidentified sp. 2 (isolate 1-3-2)	18	4	3	0	0	0
Others‡	6	5	3	0	1	2

† Microwave heating for 4 minutes. ‡ Fungi that would not sporulate and could not be identified.

TABLE 4-7. Fresh shoot weights and heights of soybean plants in soil with different treatments or from different sources.

Soil treatment	Fresh shoot weight (g/plant)		Plant height (cm)	
	1st planting	2nd planting	1st planting	2nd planting
Experiment 1				
Nematode added (N+):				
Aut	1.7 a	0.7 a	24 a	18 a
MW6	1.8 a	0.3 b	26 a	15 b
MW4	1.8 a	0.6 a	24 a	17 ab
Unt	1.0 b	0.2 b	18 b	16 ab
Mean	1.6	0.4	23	16
No nematode added (N-):				
Aut	3.2 a	3.5 a	35 a	41 a
MW6	2.8 a	3.6 a	37 a	44 a
MW4	3.2 a	3.5 a	35 a	44 a
Unt	2.9 a	1.6 b	31 a	31 b
Mean	3.0	3.1	34	40
N + vs N-	**	†	**	†
Experiment 2 (untreated soil mixed with soil treated with MW4)				
Nematode added:				
0% Unt soil	1.9 a	0.6 a	24 a	17 a
25% Unt soil	1.1 b	0.3 b	19 b	18 a
50% Unt soil	1.3 b	0.2 b	20 b	17 a
75% Unt soil	1.3 b	0.3 b	19 b	16 a
100% Unt soil	1.0 b	0.2 b	18 b	16 a
Experiment 3				
Nematode added (N+):				
FMT	1.2 a		24 a	
Unt	0.8 b		20 b	
Mean	1.0		22	
No nematode added (N-):				
FMT	2.8 a		36 a	
Unt	2.1 b		30 a	
Mean	2.4		32	
N + vs N-	**		**	
Experiment 4				
Unt soil with nematode added:				
Soil 1	7.0 c		19 b	
Soil 2	14.1 b		22 b	
Soil 3	22.6 a		24 b	
Soil 4	14.4 b		23 b	
Soil 5	16.2 ab		29 a	
Mean	14.7		23	

TABLE 4-7. (continued).

Soil treatment	Fresh shoot weight (g/plant)		Plant height (cm)	
	1st planting	2nd planting	1st planting	2nd planting
Experiment 4				
MW4 soil with added nematodes:				
Soil 1	28.1 a		25 a	
Soil 2	14.9 bc		22 b	
Soil 3	11.2 c		22 b	
Soil 4	17.2 b		22 b	
Soil 5	15.9 b		24 ab	
Mean	17.4		23	
Unt vs MW4	†		†	

Data are means of 10, 5, 10 and 6 replicates in experiments 1, 2, 3 and 4, respectively. Aut, MW4, MW6, FMT and Unt represent soil treatment with autoclaving, microwave heating for 4 minutes, microwave heating for 6 minutes, formalin, and untreated, respectively. Soil 1 was collected from a soybean field at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida; soil 2 from a soybean breeding field on campus of the University of Florida; soil 3 from a peanut breeding field; soil 4 from a fallowed field in the same farm as soil 1; and soil 5 from a fallowed field near Williston, Levy County, Florida.

The same letter in columns indicate no significant differences at $P \leq 0.05$ according to Duncan's multiple-range test. ** indicates significant difference at $P \leq 0.05$ according to ANOVA. † indicates significant interaction between nematode and soil treatments in experiment 1 or between soil treatment and soil source in experiment 4.

suppression of SCN in experiment 1-3. In experiments 1 and 2, the recolonization of soil treated with microwave heating for 4 or 6 minutes or autoclaved soil by *A. dactyloides* interfered with the detection of any nematode population suppression by fungi in untreated soil. The variation of suppression of SCN by these fungi in different experiments may have resulted from varying conditions in the greenhouse. Also the inoculum rate that was about 700 females and cysts/1.5 kg of soil in each pot may have been too high.

In experiment 4, correlation data and isolation of fungi suggest that the nematode density was suppressed by fungi in untreated soil collected from five different sites. The fungal species isolated from cysts and eggs were similar among all five soils tested (Table 4-6). The major species of fungi isolated from eggs, except a species of *Humicola*, were among the common fungal species isolated from cysts in the soybean field. The number of fungal species isolated, however, was much lower from eggs than from cysts (Chapter 3). Some fungi that were encountered at a high frequency in the cysts, such as *N. vasinfecta*, were found with a low frequency in eggs. A species of Chytridiomycetes was found in cysts and eggs by observation directly under the microscope, but it was not isolated by the methods used. The total frequency of fungi recovered from eggs was also much lower than frequency of the fungi observed directly under the microscope. More diseased eggs than healthy eggs probably were broken and lost during the preparation of egg suspensions. The sample size may need to be increased in order to recover more fungal species from the eggs.

Because the fungi colonizing the cysts and eggs are opportunistic, many fungi

that recolonize treated soil were able to colonize the cysts and eggs. Cysts and eggs extracted from soil were never observed to be completely free of fungi under the system used (Tables 4-2 to 4-6).

One fungus that was frequently observed in this series of tests was *A. dactyloides*. This fungus has not been reported previously as an egg-parasite or predator on SCN.¹³³ Because it is only moderately pathogenic to eggs of SCN (data presented in Chapter 5), the predation on J2 was probably important in the suppression of nematode population densities in soil treated with microwave heating for 4 or 6 minutes or autoclaved soil by the fungi in experiments 1-2. *Arthrobotrys dactyloides* is a poor competitor in field soil.²⁹⁷ When it was added into field soil, fungistasis was observed and this fungistasis was removed by autoclaving the soil.²⁹⁷ As little as 25% untreated soil mixed with soil treated with microwave heating for 4 minutes was enough to prevent *A. dactyloides* from recolonizing nematode cysts (Table 4-3).

Although this study shows that fungi colonizing cysts and eggs can affect the population development of SCN, the role of each individual species and the mechanism of effect of these fungi on the nematode are yet to be determined. Further study will be needed to determine the pathogenicity, the mode of infection and the factors regulating the development of these fungi.

CHAPTER 5
PATHOGENICITY OF FUNGI ISOLATED FROM
A FLORIDA SOYBEAN FIELD TO *HETERODERA GLYCINES* EGGS

Introduction

At least 168 species of fungi have been isolated from cysts of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe (Chapter 1, Table 1-2), but only a few of them have been tested for their parasitism or pathogenicity to the nematode.^{160,256,311} Godoy et al.¹⁶⁰ studied parasitism of 14 fungal species on eggs of *H. glycines* and *Meloidogyne arenaria* (Neal) Chitwood. They showed that *Verticillium lamellicola*, (F. E. V. Smith) Gams, *V. leptobactrum* Gams, *Phoma macrostoma* Mont., and *P. multistrostrata* (Mathur, Menon & Thirum) Dorenb. & Boerema parasitized high percentages of eggs of both nematodes. *Chaetomium indicum* Corda, *Fusarium solani* (Mart.) Sacc., *Fusarium oxysporum* Schlecht., *Neocosmospora vasinfecta* E. F. Smith, and *Thielavia terricola* (Gilman & Abbott) Emmons did not parasitize eggs of either nematode. *Dictyochaeta heteroderae* (Morgan-Jones) Carris & Glawe (synonym = *Codinæa heteroderae* Morgan-Jones) and *Stagonospora heteroderae* Morgan-Jones showed a low degree of parasitism on both nematodes. Meyer et al.³¹¹ assayed 22 fungi in vitro for their antagonism to eggs of *H. glycines*. Only *Phoma chrysanthemicola* Hollos and one strain each of *Verticillium chlamydosporium* Goddard and *Verticillium lecanii* (A. Zimmermann) Viégas were shown to have antagonistic effects on eggs of *H.*

glycines. Two strains of an unidentified fungus, designated as ARF18, were tested for their infectivity of SCN eggs on water agar in petri dishes.²⁵⁶ Isolate ARF18-A infected 89% of the eggs in yellow females and 61% of the eggs in brown cysts. Isolate ARF18-B infected 51% of the eggs of yellow females and 57% of the eggs of brown cysts.

More than 40 species of fungi have been isolated from females, cysts, and eggs of SCN collected from a soybean field located at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida,⁵² and from soil in pot tests in a greenhouse (Chapter 4). The objective of this investigation was to evaluate the pathogenicity to SCN eggs of selected fungi that were encountered in females, cysts, and eggs of the nematode at relatively high frequencies.

Materials and Methods

Water agar test

Twenty-one isolates of 18 species were tested by exposing them to cysts on water agar culture. The fungi included *Arthrobotrys dactyloides* Drechsler, *Cladosporium* sp., *Dictyochaeta coffeae* (Maggi & Persiani) Cabello & Arambarri, *D. heteroderae*, *Exophiala pisciphila* McGinnis & Ajello, *F. oxysporum*, *Fusarium solani* (Mart.) Sacc., *Gliocladium catenulatum* Gilm. & Abbott, *N. vasinfecta*, *Paecilomyces lilacinus* (Thom) Samson, *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker & Larson, *S. heteroderae*, *V. chlamydosporium*, sterile fungus 1 (black, yeast-like fungus) and sterile fungus 2 (isolate 1-3-2). *Paecilomyces lilacinus* and *V. chlamydosporium* were isolated from root-knot nematode egg masses on tobacco (*Nicotiana tabacum* L.) roots collected at the

University of Florida Agronomy Farm, Green Acres, Alachua County, Florida. In addition, one isolate of *P. lilacinus* obtained from the International Potato Center, one isolate of *Hirsutella rhossiliensis* Minter obtained from Dr. B. Jaffee in the University of California, Davis, one isolate of *Hirsutella thompsonii* Fisher obtained from Dr. C. W. McCoy at the University of Florida, Citrus Experiment Station and one isolate of *Beauveria bassiana* (Bals.) Vuill obtained from Dr. D. G. Boucias at the University of Florida were included for comparative purposes.

Each fungus was first cultured on cornmeal agar for 2-3 weeks before transferring to water agar. Yellow females and light brown cysts were extracted from roots of soybean cv. Cobb grown in a greenhouse. The cysts were surfaced disinfested with 0.5% NaOCl for 3 minutes and placed on water agar. Cysts that showed no signs of fungal and bacterial growth were used. Twelve to 15 fungus-free females or cysts were transferred to each petri dish. Each fungal isolate was replicated three times and one group of the nematodes was plated on cornmeal agar without fungi to serve as an untreated control.

At 3 weeks after incubation at 24 C, cysts were removed, placed on slides, covered with a cover glass, ruptured by pressing the cover glass, and observed under a light microscope. The percentage of cysts colonized and percentage of eggs parasitized by each fungus were recorded. The index of fungal parasitism of eggs was recorded on a scale from 0-10: 0 = no eggs parasitized, 1 = 1-10% eggs parasitized, 2 = 11-20% eggs parasitized, up to 10 = 91-100% eggs parasitized. The eggs were collected, suspended in water, and counted, before placing them on a 1-cm-diameter sieve with 35-

μ openings. The sieves with eggs were placed in 3-ml tissue culture wells containing 4 mM ZnCl_2 solution for hatching. The nematodes that hatched were counted at days 1, 4, 7, and 14, and at each time the hatching solution was replaced by a new one. The percentage of eggs hatched within 14 days was recorded. The test was repeated and the data presented are the average of two tests.

Greenhouse test

The fungi tested in the greenhouse included *F. oxysporum*, *H. rhossiliensis*, *P. lilacinus*, *S. heteroderae*, *V. chlamydosporium*, and sterile fungus 1 (black, yeast-like fungus). The fungi were cultured on corn grits (100 g of corn grits, 200 g of dry sand and 50 ml of water). The culture medium was autoclaved twice, inoculated with each fungus, and incubated at 24 C for 3-4 weeks. Soil taken from the soybean field was treated by heating 1-kg lots of soil placed in even layers in open plastic bags in a microwave oven (650 Watts) for 4 minutes. The treated soil was placed in autoclaved pots (10 cm high $\times$ 10 cm in diameter), and treated with fungi and nematodes according to a 2×2 factorial design: 1) corn grits with fungus + nematodes, 2) corn grits without fungus + nematodes, 3) corn grits with fungus, or 4) corn grits only. Each pot received 5 g of corn grits with or without fungi and each treatment was replicated six times.

Soybean seeds and second-stage juveniles (J2) were obtained by the procedure described in Chapter 4. Two seeds were planted in each pot on 15 December 1993, and after 1 week the seedlings were thinned to provide one per pot. Two weeks after planting, each pot received 2,000 J2 that were added to three holes about 1 cm in diameter and 2-3 cm deep around each plant. The test was maintained in a greenhouse

that had been scrubbed and washed down with 0.5% NaOCl. The pots were randomly arranged on a bench and carefully watered as needed. The plants, bench and floor of the greenhouse were sprayed as needed with insecticides, miticides and fungicides to reduce pests and diseases. Fertilizer was applied at 3, 4, 5, and 6 weeks after planting at a rate of 0.5 g of 20-20-20 (N-P-K) per pot. The fertilizer was autoclaved before being added to the pots. The lowest and highest daily temperatures (mean $\pm$ SD) in the greenhouse were 9.7 ± 6.2 and 30.8 ± 3.5 , 13.0 ± 6.3 and 31.0 ± 3.2 , 17.9 ± 2.1 and 35.5 ± 2.7 , and 17.5 ± 0.9 and 34.1 ± 5.5 , in late December 1993 and January, February, and early March 1994, respectively.

Three months after planting, the soybean plant heights and shoot weights were measured. A soil and root sample from one half of each pot was taken, and cysts and J2 were extracted with a modified centrifugal-flotation technique (Chapter 4). The densities of J2 and cysts per 100 g of soil and densities of eggs per g of soil were recorded. Eggs were extracted from cysts and counted. Fungal colonization of cysts and fungal parasitism of eggs were determined as described for the test on water agar. Cysts were collected from each treatment to determine the frequency of colonization of cysts by the original fungus and other fungi that may contaminate the soil. The cysts were washed with sterilized water, surface disinfested with 0.5% NaOCl for 3 minutes, rinsed three times with sterile deionized water, and suspended in a solution of 100 mg of streptomycin and 50 mg of chlortetracycline per liter of water. The surface disinfested females and cysts were transferred to water agar (five cysts/dish) and incubated at room temperature (23-24 C). The fungi were identified directly on the water agar and their

frequencies were recorded, except for some species that could not be identified.

The percentages of eggs parasitized and indices of parasitism of eggs in the agar test were compared for each fungus with the control by LSD ($P = 0.05$). In the greenhouse test, the percentages of fungal colonization of eggs and cysts were transformed to arcsin (x) values, and the nematode densities were transformed to $\log_{10}(x + 1)$ values before being subjected to analysis of variance (ANOVA) to compare each fungus with the untreated control. Linear regressions were performed to determine the relationship between fungal colonization of eggs and hatching rate in agar test and the relationship between nematode density and fungal colonization of cysts and eggs in the greenhouse test.

Results

Water agar test

The pathogenicity of fungi tested on water agar varied among species (Table 5-1). Of the fungi examined, *Verticillium chlamydosporium*, *P. terrestris*, sterile fungus 1 (black, yeast-like fungus), and sterile fungus 2 were most pathogenic to SCN eggs. *Verticillium chlamydosporium* parasitized about 73% of eggs, leaving them yellow to light brown in color. Fifty-six percent of the eggs were parasitized by the sterile fungus 2. *Pyrenochaeta terrestris* and the sterile fungus 1 also parasitized more than 40% of the eggs within cysts. *Pyrenochaeta terrestris* produced red pigments and some eggs parasitized by this fungus were red-colored. *Arthrobotrys dactyloides*, *E. pisciphila*, *F. oxysporum*, *F. solani*, *N. vasinfesta*, *P. lilacinus*, and *S. heteroderae* exhibited a

TABLE 5-1. Parasitism of eggs and its effect on hatching of second-stage juveniles from eggs of *Heterodera glycines* by fungi isolated from cysts of *H. glycines* from a Florida soybean field.

Fungal species	Index of parasitism of eggs	Percentage of eggs parasitized	Percentage of eggs hatched within 14 days	Percentage reduction of hatching rate
<i>Arthrobotrys dactyloides</i> Drechsler	3.9	36.0	20.6	50.1
<i>Bauveria bassiana</i> (Bals.) Vuill.	1.0	8.6	29.0	36.1
<i>Cladosporium</i> sp.	0.2	1.5	39.2	12.6
<i>Dicophaeta heteroderae</i> (Morgan-Jones) Carris & Glawe	0.7	5.5	22.4	43.8
<i>Dicophaeta coffeae</i> (Maggi and Persiani) Cabello & Aramburi	0.6	4.9	24.6	34.6
<i>Exophiala pisciphila</i> McGinnis & Ajello	2.4	20.3	24.7	50.1
<i>Fusarium oxysporum</i> Schlecht.	3.7	33.2	25.6	43.6
<i>Fusarium solani</i> (Mart.) Sacc.	2.2	16.5	18.5	52.6
<i>Glomeridium catenulatum</i> Gilm. & Abbott	0.3	2.4	20.5	52.8
<i>Hirsutiella rhossiliensis</i> Minter	0.8	7.4	24.1	41.1
<i>Hirsutiella thompsonii</i> Fisher	0.6	2.5	39.0	39.0
<i>Necosporospora vasinfacta</i> E. F. Smith:	isolate 1			
	isolate 2	18.0	21.3	55.6
	isolate 3	16.9	26.1	32.6
<i>Paeclomyces lilacinus</i> (Thom) Samson:	2.2	20.1	20.3	55.4
	Peruvian strain	31.2	36.3	21.4
	Florida strain	24.4	33.2	23.2
<i>Pyrenochaeta terrestris</i> (Hansen) Gorenz,	2.8	24.4	33.2	23.2
<i>Stagonospora heteroderae</i> Carris, Walker & Larson	4.9	45.8	12.0	60.0
<i>Stagonospora heteroderae</i> Carris, Glawe & Morgan-Jones	3.3	29.0	20.5	47.8
<i>Verticillium chlamydosporium</i> Goddard	7.6	73.4	12.8	73.8
Sterile fungus 1 (black, yeast-like fungus)	4.7	42.8	26.3	42.1
Sterile fungus 2 (isolate 1-3-2)	6.0	56.0	11.2	73.3
Control (without fungus)	0	0	46.8	
LSD $P = 0.05$	1.0	10.0	18.0	38.0

Data are means of two tests, each with three replicates for the index of parasitism and the percentage of eggs parasitized, and one replicate for the hatching rate and the percentage reduction of hatching rate. Index of fungal parasitism of nematode eggs: 0 = no eggs parasitized, 1 = 1-10% of the eggs parasitized, 2 = 11-20% of the eggs parasitized, and up to 10 = 91-100% of the eggs parasitized. The percentage reduction of hatching rate = $100 \times (\text{hatching rate in fungal treatment} - \text{hatching rate in control}) \div \text{hatching rate in control}$.

moderate degree of parasitism of eggs. The remaining species, showed a low degree of parasitism or no parasitism of eggs (Table 5-1).

All the fungi inhibited hatching of J2 from eggs (Table 5-1). *Verticillium chlamydosporium* and the sterile fungus 2 reduced the hatching rate by more than 70%. The fungi that showed little or no parasitism of eggs also inhibited hatching. The other species reduced hatching rate 20-60%. The hatching rate was correlated negatively with fungal parasitism (Fig. 5-1).

Greenhouse test

Nematode population. The nematode densities in all pots were low. The densities of cysts and eggs in soil treated with *H. rhossiliensis* and *V. chlamydosporium* were lower than that in control soil (Table 5-2). In fact, no nematodes were found in any pot treated with *H. rhossiliensis* or in four of six pots treated with *V. chlamydosporium*. The nematode densities in soil treated with the remaining fungi were not different from that in control soil (Table 5-2).

Plant growth. No differences were observed between plants treated with nematodes or those without nematodes (Table 5-3). The plant heights and fresh shoot weights in soil treated by every fungus, however, were greater than that in control soil (Table 5-3). No interaction effects on plant growth were observed between nematode and fungal treatments.

Fungal colonization of cysts and eggs. The fungal colonization of cysts and eggs by *H. rhossiliensis* and *V. chlamydosporium* was not determined because no or only few cysts were recovered from the soil treated with these two fungi. The percentages of

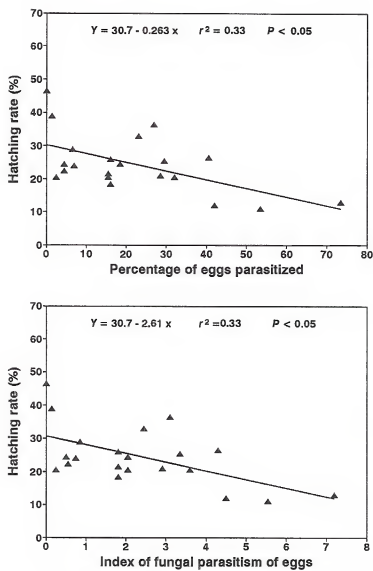


Fig. 5-1. Relationship between hatching rate of *Heterodera glycines* and fungal parasitism of eggs.

TABLE 5-2. Densities and fungal colonization of cysts and eggs of the soybean cyst nematode, *Heterodera glycines*, in soil inoculated with the nematode and nematophagous fungi in a greenhouse.

Fungi	Second-stage juveniles/ 100 g soil	Cysts/ 100 g soil	Eggs/ g soil	Percentage of cysts colonized by fungi	Percentage of eggs parasitized by fungi	Index of fungal parasitism of eggs
<i>Fusarium oxysporum</i>	2	22	22	95 *	69 *	7.1 *
<i>Hirsutiella rhossiliensis</i>	0 *	0 *	0 *	-	-	-
<i>Paeclomyces tilacinus</i>	34	29	29	97 *	70 *	7.0 *
<i>Stagonospora heteroderæ</i>	7	20	24	94 *	57 *	4.6 *
<i>Verticillium chlamydosporium</i>	1	19 *	25 *	-	-	-
Sterile fungus 1 (black, yeast-like fungus)	4	44	47	97 *	64 *	6.8 *
Control (no fungus added)	4	68	80	77	24	2.5

Data are means of six replicates, except that the percentage data for *S. heteroderæ* are five replicates. The nematode densities were transformed to $\log_{10} (\bar{x} + 1)$ values, and the percentages were transformed to arcsin ($\bar{x}$) values before being subjected to ANOVA. The ANOVA were performed to compare each fungus with the control (without fungus). * indicates significant difference from control.

TABLE 5-3. Fresh shoot weights and heights of soybean plants growing in soil containing nematophagous fungi and other infested with the soybean cyst nematode or not infested in a greenhouse.

Fungi (Fu)	Fresh shoot weight (g/plant)						Plant height (cm)			
	Nematode (N)			ANOVA			Nematode		ANOVA	
	+ -		Fu	N	Fu × N		+	-	F	N × N
	+	-								
<i>Fusarium oxysporum</i>	3.7	2.7	***	NS	NS		16.6	15.0	***	NS
<i>Hirsutiella rhossiliensis</i>	2.1	2.2	*	NS	NS		13.2	11.7	*	NS
<i>Paeclomyces tilacinus</i>	2.7	3.1	***	NS	NS		12.9	13.4	***	NS
<i>Stagonospora heteroderæ</i>	2.3	3.2	***	NS	NS		11.6	13.4	NS	NS
<i>Verticillium chlamydosporium</i>	4.8	4.7	***	NS	NS		16.2	15.4	***	NS
Sterile fungus 1 (black, yeast-like fungus)	1.5	1.8	*	NS	NS		10.8	11.1	NS	NS
Control (no fungus added)	0.9	1.0					9.3	11.4		

Data are means of six replicates. "+" means nematodes added, and "-" means without addition of nematodes. ANOVA were performed to compare each fungus with control (without fungus). Fu × N = interaction between nematode and fungus. *, ***, *** indicate significant differences at $P \leq 0.05$, 0.001 respectively. NS = not significant at $P \leq 0.05$.

cysts colonized by fungi in soil treated with *F. oxysporum*, *P. lilacinus*, *S. heteroderae*, or the black, yeast-like fungus were higher than that in control soil, although as high as 77-86% of cysts in control soil were colonized (Tables 5-2 and 5-4). The fungi recovered from cysts, however, differed among the treatments (Table 5-4). In soil receiving *F. oxysporum*, the fungus was recovered from 100% of cysts plated on water agar. Sixty-seven percent of cysts in soil treated with *P. lilacinus* were colonized, and the remaining cysts were colonized by *F. oxysporum*, *Chaetomium cochliodes* Pall., and *Curvularia lunata* (Wakker) Boedijn. In soil treated with *S. heteroderae*, 92% of cysts were colonized by *S. heteroderae* and the remaining cysts were colonized by *F. oxysporum* and other fungi. In the soil treated with the black, yeast like fungus, 37% of cysts were colonized by the fungus; 26% of cysts were colonized by *C. cochliodes* and 19% by other fungi. Although no fungus was added to the control soil, 86% of cysts were colonized by fungi. The fungi recovered from the cysts in control pots included *Trichoderma lignorum* (Tode & Harz) (18%), *F. oxysporum* (18%) and other fungi (50%) (Table 5-4). The percentages of eggs parasitized by fungi and indices of parasitism of eggs in soil treated with *F. oxysporum*, *P. lilacinus*, *S. heteroderae*, or the sterile fungus 1 were higher than that in the control (Table 5-2).

Correlation between nematode population and fungal colonization of cysts and eggs. Nematode density was correlated with the fungal parasitism of eggs, but not the percentage of cysts colonized by the fungi (Table 5-5). The correlation, however, was probably underestimated because pots in which no cysts and eggs were recovered were not included for the estimation of the correlation.

TABLE 5-4. Frequencies of fungi recovered from cysts collected from soil infested with nematophagous fungi.

Fungus treatment	Number of cysts examined	Total	Percentage of cysts colonized					
			Original fungus	<i>Fusarium oxysporum</i>	<i>Trichoderma lignorum</i>	<i>Chaetomium cochliodes</i>	<i>Curvularia lunata</i>	Other fungi
<i>Fusarium oxysporum</i>	30	100	100	100	0	0	0	0
<i>Hirsutiella rhossiliensis</i>	0							
<i>Paeclomyces tilacinus</i>	27	100	67	4	0	15	15	0
<i>Stagonospora heteroderæ</i>	39	100	92	5	0	0	0	3
<i>Verticillium chlamydosporium</i>	0							
Sterile fungus 1 (black, yeast-like fungus)	43	81	37	0	0	26	0	19
Control (no fungus added)	28	86		14	18	0	0	50

Table 5-5. Correlation coefficient (r) between nematode density and fungal colonization of cysts and eggs of *Heterodera glycines* on soybean grown in soil infested with nematophagous fungi.

Nematode density	Independent variable		
	Percentage of cysts colonized by fungi	Percentage of eggs parasitized by fungi	Index of parasitism of eggs
Cysts	0.17 NS	0.44 *	0.46 *
Eggs	0.17 NS	0.54 **	0.55 **
J2	0.09 NS	0.11 NS	0.13 NS

$n = 41$. * and ** indicate significance at $P \leq 0.05$ and 0.01 , respectively. NS = not significant.

Discussion

The improvement of plant growth in the fungal treatments could not be attributed to control of nematodes because the overall nematode densities were low and there were no differences in growth between plants with or without nematodes. The greater plant growth in fungus-treated pots may be attributed to either decomposition of the carriers, which may have provided nutrients, or fungi, such as *P. lilacinus*,²⁷⁹ producing substances that stimulated plant growth.

All cysts on water agar exposed to fungi were colonized, regardless of the pathogenicity to the nematode. In the greenhouse study, the percentage of cysts colonized by fungi varied among fungal species. Although the black, yeast like fungus was recovered at a relatively high frequency from cysts in the soybean field⁵² and other sites (Chapter 4), this fungus only colonized 37% cysts in the pots to which the fungus was added. Probably the low rate of growth of this fungus (data not shown) allowed other faster colonizing fungi to compete better than if in pasteurized soil. In contrast, all cysts in pots receiving *F. oxysporum* were colonized by the fungus. Some cysts in pots treated with *P. lilacinus* and *S. heteroderae* were also colonized by fungi other than those added. Eight-six percent of the cysts were colonized by fungi in control pots and some fungi in the control pots were pathogenic to eggs (Table 5-4).

The fungi with little or no parasitism of eggs inhibited hatching. Although the hatching rate was correlated negatively ($r = -0.58$) with percentage of eggs parasitized by the fungi or the parasitic index, the coefficient of determination (r^2) was low (0.33) (Fig. 5-1). In other words, fungal parasitism only explained about 33% of the variation

of hatching rates; consequently, other factors may also be involved in reduction of the hatching rate. Several fungi have been shown to produce metabolites that kill eggs or inhibit hatching of juveniles from the eggs.^{41,70,139,176,253,268} It is possible that this mechanism was also important in reduction of the hatching rate of SCN.

The pathogenicity of fungi does not seem to correspond to the frequency of occurrence in cysts in natural soil. Although *V. chlamydosporium* was highly pathogenic to SCN, this fungus was recovered from only one of 467 egg masses of root-knot nematode in a tobacco field (unpubl. data) near the field infested with *H. glycines*. However *V. chlamydosporium* was not isolated from the cysts of *H. glycines* in this field⁵² or from another location nearby.³²⁶ This species has been reported as a major fungal parasite of *Heterodera avenae* Woll. in Europe.^{28,232,243,246} *Verticillium chlamydosporium* does not appear to be an important parasite of cyst nematodes in subtropical climates, regardless of its pathogenicity to nematodes; however, the fungus needs further study. *Paecilomyces lilacinus* isolates have been tested widely for control of root-knot nematodes and cyst nematodes (Chapter 1). Both Floridian and Peruvian strains of *P. lilacinus* are only moderately pathogenic to SCN, although variation of virulence to nematodes was reported previously among strains.^{36,94,144,396,412,454,502} This fungus was recovered at a high frequency rate in egg masses of root-knot nematode in the tobacco field (unpubl. data), but it was only encountered in 1 of 1,711 cysts in the soybean field.⁵² This fungus was also isolated from cysts at low frequencies in other locations.^{45,158,278,334} *Fusarium oxysporum* was encountered at high frequency rates in the soybean field,⁵² as well as in other studies.^{45,157,158,278,328} The results obtained in the

greenhouse in the present study indicated that this fungus is a good soil and cyst colonizer. Although this fungus exhibited only moderate pathogenicity to SCN, it should receive further evaluation as a potential biological control agent of SCN.

The sterile fungus 2 is a slow growing species, but it is highly pathogenic to SCN. Its frequency in cysts taken from the soybean field was not determined because it was not identified. The black, yeast-like fungus was also highly pathogenic, and parasitized both nematode eggs and J2 within cysts. The fungus was encountered at a relatively high frequency in cysts collected from natural soil.⁵² Further study is needed to determine whether or not it can be introduced to the natural soil for management of SCN.

Exophiala pisciphila shares some characteristics, such as colony features and growth rate, with the black, yeast-like fungus. This fungus was encountered in cysts at a moderate frequency rate,⁵² but it is less pathogenic to the nematode eggs than is the black, yeast-like fungus. *Pyrenochaeta terrestris* is highly pathogenic to SCN eggs and was recovered in cysts at a high frequency rate,⁵² however, this fungus is considered a plant pathogen. When it was introduced to soil in the greenhouse for control of SCN, the soybean plants became diseased and died; thus no work was done with the species. *Hirsutiella rhossiliensis* is an endoparasite of vermiform nematodes,^{54,131,198,199,204} including J2 of *H. avenae*⁴⁴⁹ and *Heterodera schachtii* Schmidt.²⁰⁰ On agar this fungus was observed to attack J2 of SCN, but it has little pathogenicity to eggs (Table 5-1). Some studies have shown that *H. rhossiliensis* is a poor soil competitor.²⁰⁵ In natural soil the population of this fungus develops slowly and is nematode density dependant.¹⁹⁶ In the present study the fungus suppressed nematode population densities completely in

pasteurized soil. Whether this fungus can be used for control of SCN needs further study in natural soil, both in the greenhouse and field plots.

The three isolates of *N. vasinfecta* varied in color of colony and perithecia. The fungus was encountered in cysts with the highest frequency.⁵² The pathogenicity of the three isolates appeared to be similar at moderate levels. The frequency of *F. solani* in cysts⁵² and its pathogenicity to eggs were similar to that of *N. vasinfecta*. *Dictyochaeta heterodera* was shown to have a low degree of parasitism on eggs.¹⁶⁰ The present study agrees with this result. The first observation of *Dictyochaeta coffeae* in cysts was made in the present study. This fungus is similar to *D. heterodera* in pathogenicity to eggs. Although these two fungi are not highly pathogenic to eggs, they were recovered from cysts with a relatively high frequency.⁵² The isolate of *S. heteroderae* used in the present study was moderately pathogenic to the eggs, whereas an isolate of this fungus was shown to have a low degree of parasitism on the nematode in a previous study.¹⁶⁰ This fungus was encountered in cysts in natural soil with a relatively high frequency.^{52,371} *Beauveria bassiana* and *H. thompsonii* are insect parasites and did not appear to be pathogenic to SCN. *Cladosporium* sp. which was isolated from aphids on soybean plants in the greenhouse was not pathogenic to SCN. In the present study, it was observed that eggs containing J2 were generally resistant to fungal attack. This observation supports a previous report.¹⁹⁵ The percentage of eggs parasitized by fungi in the greenhouse was higher than that of the corresponding fungi on agar. The yellow females and light brown cysts used in agar tests may have contained more developed eggs than did the females on roots. Yellow females that are 30-35 days old and contain eggs of

varying maturity are probably the best to use in pathogenicity tests. Two to three weeks of incubation is optimum for observation of fungal parasitism on eggs. If the incubation period exceeds 4 weeks, some eggs, especially those parasitized by *V. chlamydosporium*, are consumed by the fungus.

CHAPTER 6 OBSERVATIONS ON FUNGAL PENETRATION OF THE CYST WALL OF *HETERODERA GLYCINES*

Introduction

Many fungi have been isolated from cysts, and some are parasites of females and eggs (Chapter 1). The modes of infection of females and cysts by fungi are poorly understood. Tribe⁴⁷⁵ speculated that routes of fungal invasion into cysts are generally through natural body openings. He stated that penetration through the hardened cuticle does not seem to occur. Kerry,²³⁷ however, hypothesized that, fungi may invade cyst nematodes directly through the cyst wall. Some reports show that fungi can penetrate the cyst wall.^{183,255,258} The cyst wall of *Heterodera schachtii* Schmidt was penetrated by *Verticillium lecanii* (Zimm.) Viégas 60 hours after inoculation on glass slides.¹⁸³ Kim et al.²⁵⁸ reported that a sterile fungus designated "ARF 18" could penetrate the cyst wall of *Heterodera glycines* Ichinohe, which was obtained from a pot culture in a greenhouse. However, little evidence has been shown to whether these fungi penetrate the cyst wall from outside the body cavity or from inside the body cavity.

More than 40 fungal species were isolated from females and cysts of *H. glycines* collected from a Florida soybean field⁵² (Chapters 2 and 4). Some of these fungi have been tested for their pathogenicity to the nematode eggs (Chapter 5). The objective of this study was to examine some of these fungi, which were encountered at a relatively

high frequency for their capability of penetrating the nematode cyst wall.

Materials and Methods

Fungi. The following 12 species of fungi were examined: *Arthrobotrys dactyloides* Drechsler, *Dictyochaeta heteroderae* (Morgan-Jones) Carris & Glawe, *Exophiala pisciphila* McGinnis & Ajello, *Fusarium oxysporum* Schlecht., *Gliocladium catenulatum* Gilm. & Abbott, *Hirsutella rhossiliensis* Minter, *Neocosmospora vasinfecta* E. F. Smith, *Paecilomyces lilacinus* (Thom) Samson, *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker & Larson, *Stagonospora heteroderae* Carris, Glawe & Morgan-Jones, *Verticillium chlamydosporium* Goddard, and a sterile fungus (black, yeast-like fungus). *Verticillium chlamydosporium* was isolated from an egg mass of *Meloidogyne* sp., in a Florida tobacco field. *Paecilomyces lilacinus* was obtained from the International Potato Center, Peru. *Hirsutella rhossiliensis*, an endoparasite of vermiform nematodes, was obtained from Dr. B. Jaffee, University of California Davis. The remaining species were isolated from females or cysts of *H. glycines* collected from the Florida soybean field described previously.⁵² The fungi were cultured on corn meal agar (Difco Laboratories, Detroit, MI) for 1-3 weeks before used.

Nematodes. The nematodes used were obtained from a tissue culture modified from a technique reported by Lauritis et al.²⁷² Second-stage juveniles (J2) were obtained by following the procedures described in Chapter 4 and used as inoculum for the tissue culture. Soybean seeds were washed with water, treated with 70% ethanol for 1 minute, washed with sterile water at least three times, treated with 0.001% HgCl₂ for 5 minutes,

washed with sterile water another three times, and rinsed with sterile water for at least 10 minutes. The treated seeds were placed on potato dextrose agar (Difco Laboratories, Detroit, MI) in petri dishes and incubated at room temperature (23-25 C) for 5 days. Root radices were aseptically excised about 3 cm from the root tip, and three root tips were transferred to each petri dish containing Gamborg's B-5 medium,¹⁵⁰ at pH 5.7-5.8. After 1-2 weeks, each dish received about 1 ml of suspension of J2 (about 300 J2). The cultures were maintained in the dark at 28 C. Light brown cysts that formed on roots were used for inoculation with fungi.

Inoculation of nematode with fungi. About 30 cysts were placed on a glass slide. Spore and mycelium suspensions were made for each fungus that sporulated. About 1 ml of sterile water was added onto each petri dish containing a fungal colony, and spores and mycelium were scraped with a knife in the water to make a fungal suspension. The suspension was removed with a sterile pipet and placed on the cysts. The slides with treated cysts were placed in petri dishes containing a small amount of sterile water. Cysts were added directly to fungal colonies of the sterile species and *A. dactyloides*, which had few spores. The cysts were incubated at 24 C for 12 hours to 1 month. A group of cysts without fungal treatment was included as a control.

Light microscopy observation. The cysts were examined with the aid of light microscopy at 12 hours, 1, 2, 3, 4, 7, 14 days, and 1 month following their inoculation. The cuticles of cysts were prepared by a technique modified from the technique used for preparing perineal patterns of root-knot nematodes.⁴⁶⁴ The cysts were cut in half, transversely, with a knife in water on a slide and the body contents were pulled out. The

cuticles were placed in a drop of 45% lactic acid on a slide and the body tissues that adhered to the cuticle were removed by brushing the inner surface of the cuticle with a bristle made from bamboo. The cleaned cuticle was transferred to a drop of glycerin or lactophenol, and covered with a coverslip. In order to determine whether the fungal penetration was from the outside or inside, the coverslip was placed on the cuticle in such a way that the cuticle folded inward. The direction of fungal penetration could be determined by observing the edge of the cuticle fold. By pushing the coverslip, the folded cuticle could be manipulated so that most of the cuticle could be examined. Semi-thin sections about 3-5 μm thick obtained from samples prepared for transmission electron microscopy were also prepared and viewed. Sections were stained with 0.1% toluidine blue O in 1% sodium borate or with Nile blue A and mounted in immersion oil. Photographs were taken by using Normarsky optics.

Scanning electron microscopy observation. The mycelium on the surface of cysts was removed by carefully brushing with a bamboo bristle. The cysts were cleaned by treatment of ultrasound in deionized water. Following the treatment, the cysts were fixed for 24 hours in a modified Karnovsky's fixative (2% formaldehyde + 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2).¹³⁰ The fixed cysts were rinsed two times in phosphate buffer (pH 7.2) for 20 minutes each time and post-fixed with 1% osmium tetroxide in the same buffer for 2 hours. After two rinses of 10 minutes each in deionized water, the specimens were dehydrated through an ethanol series of 30, 50, 70, 80, 90, 100, 100, and 100%, 20 minutes each. The ethanol was replaced by three changes (one change of 1:1 of ethanol:amyl acetate, and two changes of 100% amyl

acetate, 20 minutes each). The specimens were critical point dried, mounted on aluminum stubs, coated with gold and palladium, and were viewed with a 'Hitachi S-517' scanning electron microscope at 20 kV or 'Hitachi S-4000' scanning electron microscope at 10 kV.

Transmission electron microscopy observation. Specimens that were examined with transmission electron microscopy included the cysts inoculated with *Exophiala pisciphila*, *F. oxysporum*, *N. vasinfecta*, *P. lilacinus*, *P. terrestris*, *S. heteroderae*, *V. chlamydosporium*, and the sterile fungus. The specimens were fixed in the modified Karnovsky's fixative for 1 hour at room temperature, 2 hours at 67 C, and cut in half with small knife in the same fixative in a hood. The fixative was replaced with a new one and incubated at 4 C for 22 hours. The specimens were washed twice with buffer, 20 minutes each, and post-fixed with 1% osmium tetroxide for 3 hours at room temperature in the same buffer. After rinsing one time in buffer and one time in deionized water, 10 minutes each, the specimens were dehydrated in a series of ethanol: 10, 25, 50, 75, 85, 95% ethanol, 20 minutes each, and then for 30 minutes in each of changes of 100% ethanol. The ethanol was replaced by acetone with two changes, 20-30 minutes each. The specimens were infiltrated through a series of Spurr's¹³⁰ plastic: 30% (in acetone) for 12 hours, 70% for 12 hours, 100% for 12 hours, and 100% for 24 hours. Following the infiltration, the plastic was replaced with a new plastic, and the specimens were polymerized by incubation in an oven at 60 C for 48 hours. Ultrathin sections (60-100 nm) were cut with a glass knife on a LKB 8000 Ultratome III ultramicrotome or with a diamond knife on a Sorvall Porter-Blum MT2-B ultramicrotome. The sections were

mounted on formvar film on 100 mesh copper grids and stained in 1% uranyl acetate for 15 minutes and in lead citrate for 5 minutes.¹³⁰ The specimens were examined with a JEOL 100 CX transmission electron microscope at 60 kV.

Results

The fungal penetration of the *H. glycines* cyst wall is summarized in Table 6-1. Ten of 12 fungal species were found to penetrate the cyst wall (Table 6-1; Figs. 6-1 to 6-17). Penetration of the cyst wall from outside to inside, however, was observed only in four species, namely *E. pisciphila* (Fig. 6-3C), *F. oxysporum* (Fig. 6-4B), *P. terrestris* (Figs. 6-8B,C, 6-10A), and the black, yeast-like fungus (Figs. 6-16A,B). The earliest time when penetration was observed after exposure of the cysts to fungi was 1 day for *F. oxysporum* and *P. terrestris*; 2 days for *E. pisciphila*, *P. lilacinus* and *S. heteroderae*; 3 days for *V. chlamydosporium*, 4 days for *A. dactyloides*; and 7 days for *D. heteroderae*. No fungal penetration was observed on cysts exposed to *G. catenulatum* or *H. rhossiliensis* for up to 1 month. The cyst wall without fungal penetration is illustrated in Fig. 6-18.

Generally, penetration could be completed by a single hypha. After mycelium on the surface of the cyst wall was removed, some penetration hyphal pegs either remained on the cuticle or were removed along with the mycelium, leaving circular holes in the wall (Figs. 6-5, 6-7B, 6-9, 6-11B, 6-13). The penetration hyphal pegs within the cyst wall were generally less than 1 μm (usually about 0.5 μm) in diameter, which is much less than the diameter of a regular hypha. Numerous organelles were observed in

TABLE 6-1. Summary of penetration of *Heterodera glycines* cyst wall by fungi.

Fungal species	Penetration from inside to outside	Penetration from outside to inside	Time for penetration (day) †
<i>Arthrobotrys dactyloides</i>	Yes	?	4
<i>Dictyochoaeta heteroderae</i>	Yes	?	7
<i>Exophiala pisciphila</i>	Yes	Yes	2
<i>Fusarium oxysporum</i>	Yes	Yes	1
<i>Gliocladium catenulatum</i>	No	No	
<i>Hirsutella rhossiliensis</i>	No	No	
<i>Neocosmospora vasinfecta</i>	Yes	?	3
<i>Paecilomyces lilacinus</i>	Yes	?	2
<i>Pyrenochaeta terrestris</i>	Yes	Yes	1
<i>Stagonospora heteroderae</i>	Yes	?	2
<i>Verticillium chlamydosporium</i>	Yes	?	3
Sterile fungus 1 (a black, yeast-like fungus)	?	Limited in outer layers	4

† The earliest time when penetration was observed after exposure of cysts to fungi.

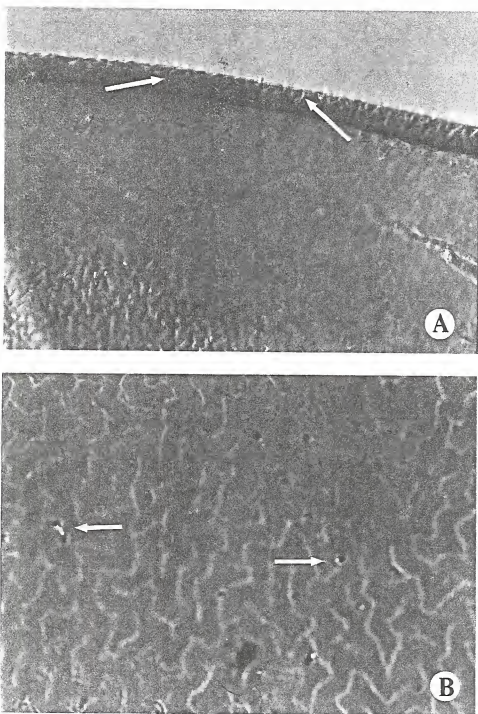


Fig. 6-1. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by *Arhrobotrys dactyloides* 7 days after inoculation. A) Hyphae (arrow) penetrated through the cyst wall ($\times 650$). B) Top view of hyphal pegs or holes in the cyst wall as a result of penetration by the fungus ($\times 1610$).

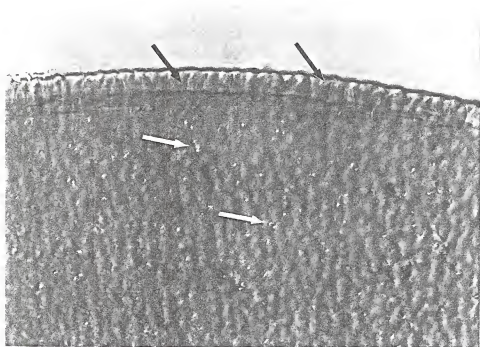


Fig. 6-2. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by *Dictyochaeta heteroderae* 7 days after inoculation. Black arrows indicate hyphae penetrating from cyst cavity towards outside, and white arrows indicate hyphal pegs or holes on the cyst wall as a result of penetration by the fungus ($\times 650$).

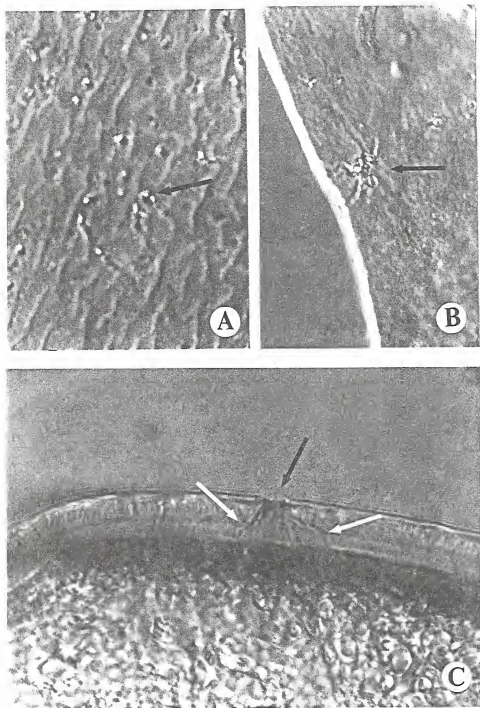


Fig. 6-3. Photomicrographs of penetration of *Heterodera glycines* cyst wall by *Exophiala pisciphila*. A) Top view of hyphal pegs or holes (arrow) in the cyst wall as a result of penetration by the fungus 43 hours after inoculation ($\times 1610$). B) A cluster of hyphae penetrating cyst wall 7 days after inoculation ($\times 650$). C) The fungus penetrated the cyst wall from outside to enter the cyst cavity 7 days after inoculation. The penetration started at a point (black arrow) on the surface of cyst wall, and several penetrating hyphae (white arrows) developed from this point ($\times 1610$).

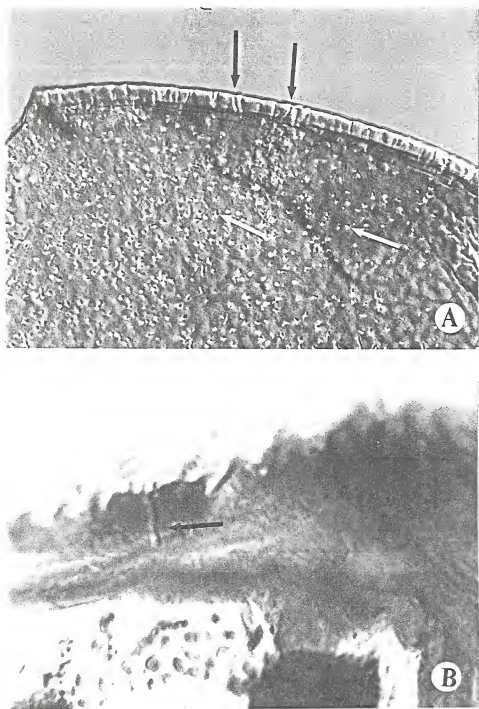


Fig. 6-4. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by *Fusarium oxysporum* 7 days after inoculation. A) Black arrows indicate the hyphae penetrating from cyst cavity towards the outside, and white arrows indicate hyphal pegs or holes as a result of penetration by the fungus ($\times 650$). B) A hypha (arrow) penetrated the cyst wall from outside towards the cyst cavity ($\times 1880$).

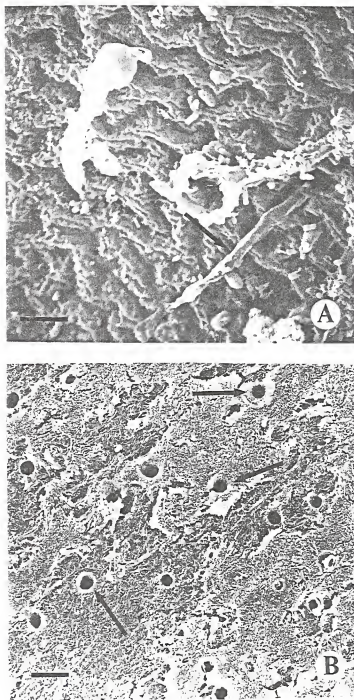


Fig. 6-5. Scanning electron micrographs of the penetration of *Heterodera glycines* cyst wall by *Fusarium oxysporum* 7 days after inoculation. Scale bars = 3 μ m. A) Hyphae (arrow) growing from cyst cavity (arrow). B) Holes (arrows) on cyst wall as a result of penetration by the fungus.

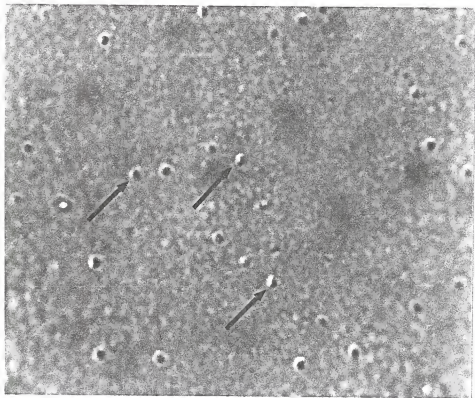


Fig. 6-6. Photomicrograph of the penetration of *Heterodera glycines* cyst wall by *Neocosmospora vasinfecta* 72 hours after inoculation ($\times 1880$). Arrows indicate hyphal pegs or holes as a result of the penetration by the fungus.

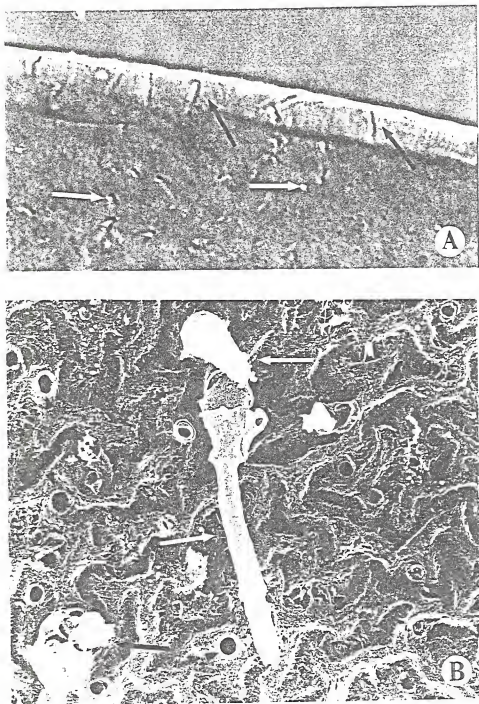


Fig. 6-7. Micrographs of the penetration of *Heterodera glycines* cyst wall by *Paecilomyces lilacinus* 7 days after inoculation. A) Photomicrograph shows hyphae (black arrows) penetrating the cyst wall from the cavity towards the outside and hyphal pegs or holes (white arrows) as a result of fungal penetration ($\times 1610$). B) Scanning electron micrograph shows hyphae growing from the cyst wall (white arrows) or hyphal pegs protruding from the cyst wall ($\times 4350$).

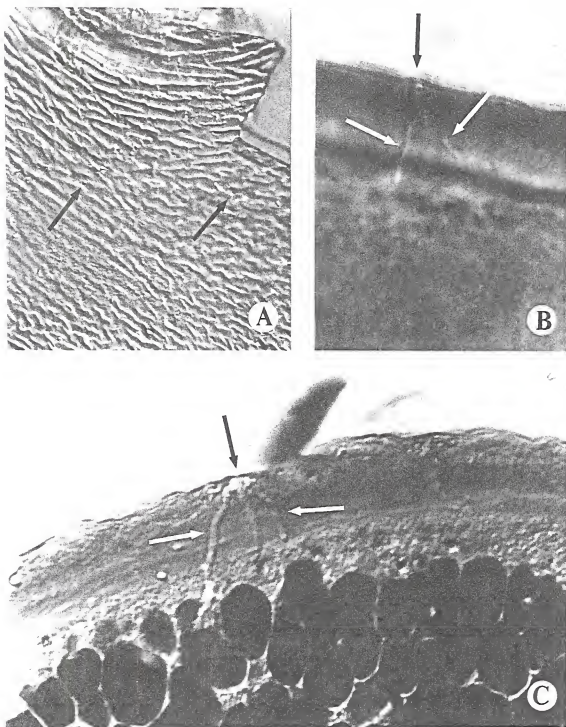


Fig. 6-8. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by *Pyrenochaeta terrestris*. A) Top view of hyphal pegs or holes (arrows) as a result of penetration by the fungus 7 days after inoculation ($\times 650$). B and C) The fungus initiated penetration at a point (black arrows) from which penetration hyphae (white arrows) developed. B) Cuticle of a cyst exposed to the fungus for 48 hours ($\times 1880$). C) Semi-thin section of a cyst exposed to the fungus for 7 days ($\times 1880$).



Fig. 6-9. Scanning electron micrographs of the penetration of *Heterodera glycines* cyst wall by *Pyrenochaeta terrestris* 7 days after inoculation. A) Arrows indicate holes as a result of fungal penetration. Scale bar = 5 μm . B) Higher magnification of the boxed area of Fig. 6-9A showing hyphal pegs (arrows) protruding from the cyst wall. Scale bar = 1 μm .

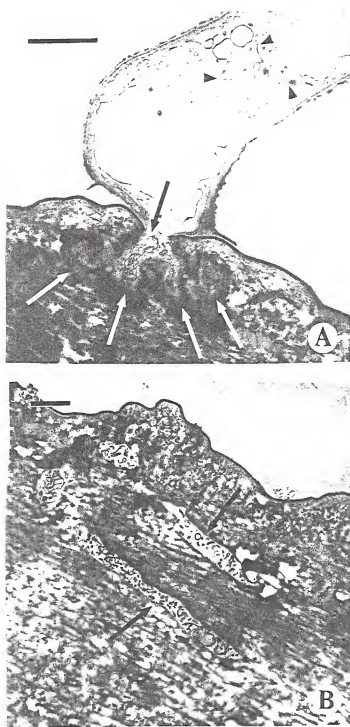


Fig. 6-10. Transmission electron micrographs of the penetration of *Heterodera glycines* cyst wall by *Pyrenochaeta terrestris* 7 days after inoculation. Scale bars = 1 μ m. A) Hypha initiated penetration on surface of cyst wall at a point (black arrow) from which several branches of plasma (white arrows), that have no definitive cell wall, developed and moved towards the inside of the cyst wall. At the arrow tips organelles can be visualized. B) Two penetration hyphae (arrows) are visible with condensed organelles within the cyst wall.

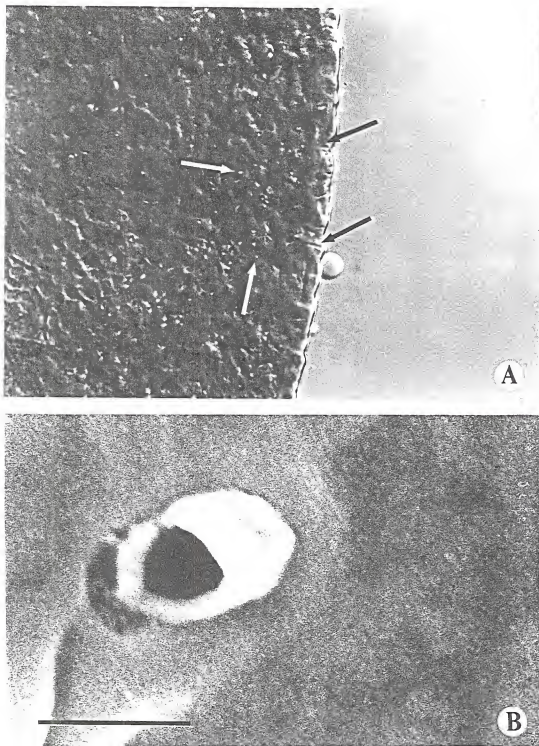


Fig. 6-11. Micrographs of the penetration of *Heterodera glycines* cyst wall by *Stagonospora heteroderae*. A) Photomicrograph shows hyphae (black arrows) growing from the cyst cavity towards the outside and top view of hyphal pegs or holes (white arrows) as a result of fungal penetration 3 weeks after inoculation ($\times 1880$). B) Scanning electron micrograph shows a hole on the cyst wall 7 days after inoculation. Scale bar = $0.5\ \mu\text{m}$.

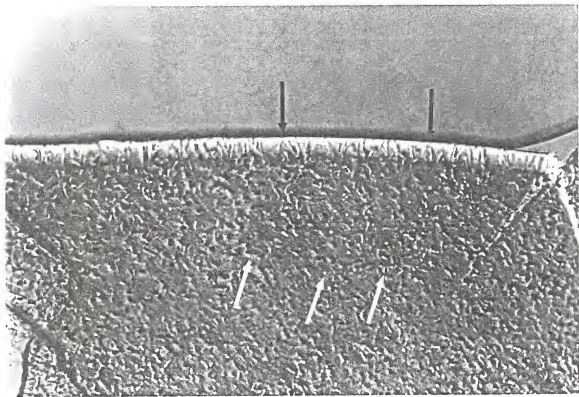


Fig. 6-12. Photomicrograph of the penetration of *Heterodera glycines* cyst wall by *Verticillium chlamydosporium* 3 weeks after inoculation. Black arrows indicate hyphae growing from the cyst cavity towards the outside. White arrows show top view of hyphal pegs or holes as a result of fungal penetration ($\times 760$).



Fig. 6-13. Scanning electron micrograph of the penetration of *Heterodera glycines* cyst wall by *Verticillium chlamydosporium* 7 days after inoculation. The two holes resulted from fungal penetration. Scale bar = 1 μ m.



Fig. 6-14. Transmission electron micrograph of the penetration of *Heterodera glycines* cyst wall by *Verticillium chlamydosporium* from cyst cavity through the cyst wall 7 days after inoculation. Scale bar = 1 μ m.

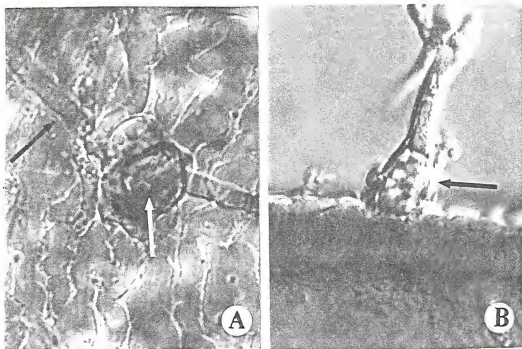


Fig. 6-15. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by a sterile fungus (a black, yeast-like fungus) 4 days (A) or 7 days (B) after inoculation. A) Top view of hyphae (black arrow) with swollen cells (white arrow) that may have attached to the cyst wall ($\times 1880$). B) Lateral view of a swollen cell (arrow) attached to the cyst wall ($\times 1880$).



Fig. 6-16. Transmission electron micrographs of the penetration of *Heterodera glycines* cyst wall by a sterile fungus (a black, yeast-like fungus) 7 days after inoculation. Scale bars = 2 μ m. A) A hypha (arrow) attached to the cyst wall. B) Hyphae initiated penetration (arrow).

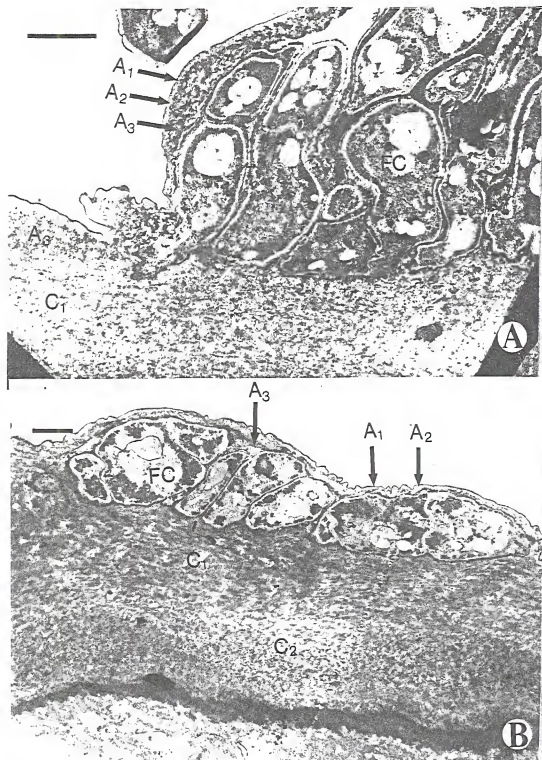


Fig. 6-17. Transmission electron micrographs of the penetration of *Heterodera glycines* cyst wall by a sterile fungus (a black, yeast-like fungus) 7 days after inoculation. Scale bars = 2 μm. A and B) show the compacted pseudoparenchyma-like cells within the cyst wall. A₁, A₂, A₃, C₁, and C₂ represent layers of the cuticle. FC = fungal cells.

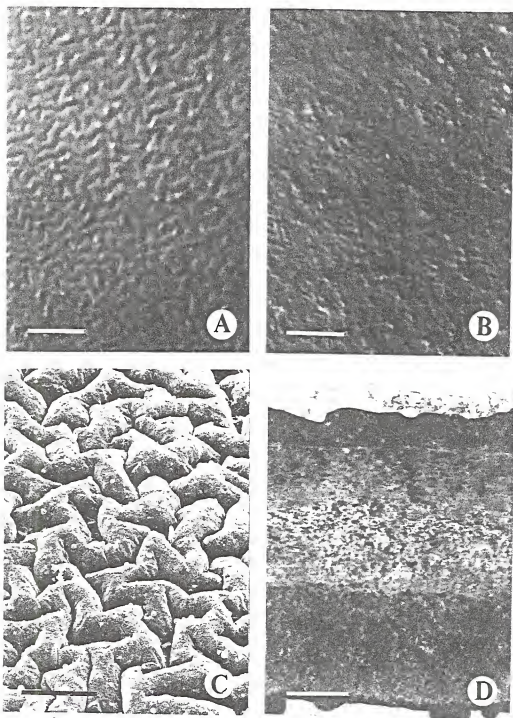


Fig. 6-18. Micrographs of *Heterodera glycines* cyst cuticle without mycelium on surface or fungal penetration. A and B) Photomicrographs show surface of cuticle ($\times 760$). C) Scanning electron micrograph shows surface of cuticle. Scale bar = $5\ \mu\text{m}$. D) Transmission electron micrograph shows section of cuticle. Scale bar = $2\ \mu\text{m}$.

penetration hyphae of *P. terrestris* (Fig. 6-10). When penetration of the cyst wall was initiated from outside to inside, a hypha attached to a point of the cyst wall where both fungal and cyst walls were dissolved. The plasma with condensed organelles moved from the fungal cell into the cyst wall, from which several branches of plasma without a definitive cell wall may have developed. The penetrating hyphae of *V. chlamydosporium* in the cyst wall, however, appeared to have cell walls (Fig. 6-14).

The black, yeast-like fungus exhibited a distinctly different mechanism of interacting with the cyst wall than did the other fungi studied. When the fungus contacted the surface of the cyst wall, hyphal cells enlarged (Fig. 6-15 A and B) and were tightly attached to the cyst wall (Fig. 6-15 B and 6-16 A). The fungus penetrated through cuticle layers A1, A2, and A3 (see reference⁴²⁸ for nomenclature of cuticular layers of the genus *Heterodera*) and proliferated between layers A3 and C (Figs. 6-15B, 6-16A,B). The fungal cells enlarged and were tightly compacted in the cuticle so that they appeared to form a pseudoparenchymatous structure. The fungus, however, was not observed penetrating through the cyst wall.

Discussion

Most fungi penetrated the cyst wall, and at least three species, *Exophiala pisciphila*, *F. oxysporum* and *Pyrenochaeta terrestris*, penetrated through the cyst wall from the outside. This suggests that direct fungal penetration of cyst walls may be common among nematophagous fungi. Although penetration of the cyst wall from outside to inside was not observed most of the fungi, additional studies, however, are

needed before the possibility can be ruled out.

The ability to penetrate the cyst wall from the outside may not be important for a fungus to colonize a cyst because fungi can readily enter cysts through natural body openings.^{237,330} In fact, fungi were usually found inside cyst cavities within 1 day after inoculation, before any direct penetration of the cyst wall was observed. Whether these fungi have the same ability to enter young females or cysts through natural body openings is yet to be determined. If the natural body openings of the young females and cysts repel penetration, then the ability of cuticle penetration may be important for the infection process to occur. If a fungus can invade young females and cysts, the fungus may have more chance to destroy eggs within the cysts because eggs in the early developmental stage are more vulnerable to fungal infection¹⁹⁵.

Penetration hyphae were often found to penetrate through the cyst cavity to the outside. In contrast, fewer hyphae were found penetrating the cyst wall from outside to inside. The reason for this is not clear. It may be that the nutrient contents within the cysts provided for a rapid increase of fungal biomass. With the internal increase in fungal biomass, there is a greater chance for fungal penetration being initiated from inside than from outside. An alternative speculation is that the chemical interaction between fungi and inner layers of the cyst wall differs from the interaction between fungi and outer layers of cyst wall.

The mechanisms involved in penetration of the nematode cuticle is not yet clear,²²⁰ although both enzymatic⁴¹⁸ and mechanical^{1220,345,488,489} mechanisms are thought to be involved. In the present study, penetration hyphae of *P. terrestris* that were

observed within the cyst wall were rich in organelles (Fig. 6-10B). This suggests an active mechanism that involves chemical activity. The penetration of the cyst wall by the black, yeast-like fungus was confined between layers A and C (Fig. 6-17). It is possible that this fungus has some enzymes that can dissolve the layer A but not layer C. Since the collagen-like protein is a main component in layer C, but not in layer A,⁴²⁸ the fungus may lack activity of collagenase. Further study, however, is needed to confirm this speculation. Diameters of penetration pegs were usually less than those of regular hyphae. This may be a mechanism that evolved for saving energy during penetration. The small penetration pegs may facilitate mechanical penetration if this mechanism is involved.

Electron microscopy revealed some details of the fungal penetration process of the cyst wall. Scanning electron microscopy, however, provides little information for detecting fungal penetration from outside to inside. Although transmission electron microscopy provides clearer illustration of the fungal penetration process than does either light microscopy or scanning electron microscopy, only a limited number of samples can be examined with transmission electron microscopy. Looking for penetrating hyphae is a tedious work and costly, and chances for finding these hyphae is small. The penetration of the cyst wall by fungi can be examined more easily by using the light microscopy procedure developed in this study. This procedure is simple and can be used to examine a relatively large number of specimens. Care must be taken, however, in order to distinguish granules or bubbles on the surface of the cuticle from fungal hyphae or penetrating holes.

CHAPTER 7

SUMMARY

In this research, the mycoflorae in females and cysts of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, were investigated in a Florida soybean (*Glycine max* L.) field, and their influences on the nematode were estimated in the laboratory and greenhouse. A total of 1,620 females and cysts were examined in 1991, and 1,303 were examined in 1992. More than 40 fungal species were isolated from the females, cysts, and eggs. In the field, the frequency of fungi colonizing white and yellow females was low, but a high frequency of fungi was encountered in brown cysts, and the frequency increased with time of exposure of the cysts to the soil. No single fungal species predominated in the nematode females or cysts in this field. Rarely was a female or cyst colonized by more than one fungus. The most common fungi isolated from the females and cysts were *Neocosmospora vasinfecta* E. F. Smith, *Fusarium solani* (Mart.) Sacc., *Fusarium oxysporum* Schlecht., *Dictyochaeta coffeae* (Maggi & Persiani) Cabello & Arambarri, *Dictyochaeta heteroderae* (Morgan-Jones) Carris & Glawe, *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker & Larson, *Exophiala pisciphila* McGinnis & Ajello, *Gliocladium catenulatum* Gilm. & Abbott, *Stagonospora heteroderae* Morgan-Jones, and a black, yeast-like fungus. The similarity index for mycoflora in brown cysts between two sampling dates was correlated negatively with the length of time between the dates. The communities of common fungal species isolated from cysts

in several regions in the southeastern United States appear to be similar.

Four experiments were conducted in the greenhouse to evaluate the influence of mycoflora on SCN population densities in soil with different treatments or from five different sites in Florida. In experiment 1, the soil was treated with microwave heating for 4 or 6 minutes, autoclaved, or left untreated. In experiment 2, the mycoflora in untreated soil was diluted by the soil treated with microwave heating for 4 minutes. In experiment 3, the soil was either treated with formalin or left untreated. In experiment 4, soil was collected from five sites and either treated with microwave heating for 4 minutes or left untreated. The response of the nematode to the mycoflora varied among experiments. The untreated soil showed little suppression of SCN in experiments 1-3. In experiment 4, however, the nematode density was suppressed by fungi in untreated soil collected from five different sites. The percentages of cysts colonized and eggs parasitized by fungi in soil treated with microwave heating were lower than in untreated soil. The nematode densities in treated soil were 4.3-fold higher for cysts, 7.1-fold higher for eggs, and 7.5-fold higher for second-stage juveniles, in comparison with untreated soil. The number of eggs produced per female was 73% higher in treated soil compared to untreated soil. The nematode population density was correlated negatively with both percentage of the cysts colonized by fungi and index of parasitism of eggs. However, no differences in nematode densities and frequencies of fungal colonization of cysts or eggs in soil were observed among the five sites.

Twenty-one isolates of 18 species of fungi were tested on water agar for their pathogenicity to the eggs within cysts. Fungal parasitism and hatching rate of the

nematode eggs were determined after exposure of the nematodes to the fungi on water agar for 3 weeks at 24 C. *Verticillium chlamydosporium* Goddard parasitized 73 % of the eggs and reduced hatching rate 74 %. *Pyrenochaeta terrestris* and two sterile fungi also parasitized a relatively high percentage of eggs and reduced hatching rates 42-73 %. *Arthrobotrys dactyloides* Drechsler, *F. oxysporum*, *Paecilomyces lilacinus* Samson, *S. heteroderae*, *N. vasinfecta*, *F. solani*, and *E. pisciphila* were moderately pathogenic to eggs (20-36 % of eggs parasitized and hatching rate reduced 21-53 %). *Beauveria bassiana* (Bals.) Vuill, *Hirsutella rhossiliensis* Minter, *Hirsutella thompsonii* Fisher, *D. heteroderae*, *D. coffeae*, *G. catenulatum*, and *Cladosporium* sp. showed low or no parasitism of the nematode eggs, but the hatching rate was also reduced by these fungi. The hatching rate was correlated negatively with the percentage of eggs parasitized. *Fusarium oxysporum*, *H. rhossiliensis*, *P. lilacinus*, *S. heteroderae*, *V. chlamydosporium*, and the black yeast-like fungus were tested further in soil in a greenhouse. Although the overall nematode densities in the pots were low, the nematode densities were lower in soil treated with *H. rhossiliensis* and *V. chlamydosporium* than in control soil. The SCN population densities were correlated negatively with the percentage of eggs parasitized, but not with the percentage of cysts colonized by the fungi.

Twelve fungal species were examined with the add of light, scanning electron, transmission electron microscopy, for their capability to penetrate the cyst wall of *H. glycines*. *Arthrobotrys dactyloides*, *D. heteroderae*, *E. pisciphila*, *F. oxysporum*, *N. vasinfecta*, *P. lilacinus*, *P. terrestris*, *S. heteroderae*, *V. chlamydosporium*, and the black, yeast-like fungus were found to penetrate of the cyst wall. Most of these

penetrated the cyst wall from the inside. At least three species, *E. pisciphila*, *F. oxysporum*, and *P. terrestris* penetrated through the cyst wall from the outside. The procedure developed in the present study for examining the fungal penetration of cyst wall by using light microscopy is easy to be used and can be employed to examine a large numbers of specimens.

From the studies of this project, the following conclusions can be made: 1) the frequency of fungi in white and yellow females was low, but a high frequency of fungi was encountered in brown cysts, and the frequency increased with time of exposure of the cysts to the soil; 2) the similarity of mycoflora in brown cysts for two sampling dates decreased with the days between the two sampling dates; 3) the mycoflorae in soil collected from five sites in Florida suppressed the SCN population under greenhouse conditions; 4) the fungi isolated from the females and cysts differed in pathogenicity to the eggs, and some fungal species showed a high pathogenicity; 5) the hatching rate of the nematode was correlated negatively with the percentage of eggs parasitized; and 6) 10 of 12 fungi studied penetrated the cyst wall and at least three species penetrate through the cyst wall from the outside.

The present studies provided some information of ecology and pathogenicity of the fungi, and modes of infection of the nematode by the fungi. Further studies, however, are needed to determine the influence of the mycoflora in natural soil and under field conditions. Several species, such as the black, yeast-like fungus, showed a relatively high pathogenicity to the nematode eggs and were encountered at a relatively

high frequency in cysts in the soybean field. It may be worthwhile to evaluate the potential of these fungi as biological control agents in natural soil, both in greenhouse and field.

APPENDIX 1
RESPONSE OF *MELOIDOGYNE* SPP. TO *PASTEURIA PENETRANS*,
FUNGI, AND CULTURAL PRACTICES IN TOBACCO

Introduction

Continuing environmental problems associated with the use of nematicides⁴⁶⁷ have resulted in a sense of urgency regarding the search for alternative nematode management tactics.²³⁹ Biological control of nematodes with microbial agents is an alternative management tactic that is receiving increased interest among nematologists. Biocontrol of plant-parasitic nematodes has been reported from several countries and on different nematodes.^{27,131,237,300,315,455} Many soil-borne fungi have been demonstrated to be antagonists of nematodes. These include predaceous fungi, endoparasites of vermiform nematodes, fungi colonizing eggs and females of sedentary endoparasitic nematodes, and fungi that are antibiotic to nematodes.⁴⁴⁸ Recently, another group of nematode antagonists, endospore-forming bacteria, especially *Pasteuria penetrans* (Thorne) Sayre & Starr, has been reported throughout the world. *Pasteuria penetrans* is a density-dependent obligate parasite of many species of nematodes, mainly root-knot nematodes, *Meloidogyne* spp. Some reports suggest that *P. penetrans* may suppress nematode population densities below economic damage levels when their densities built up over time.^{315,448} Additional knowledge about the ecology of these nematode antagonists in agricultural ecosystems, however, is needed before they can be effectively used in

nematode management (IPM) programs.

The objective of this study was to determine the effects of nitrogen fertilizer, Autumn cover treatments and tobacco (*Nicotiana tabacum* L.) cultivars on the root-knot nematodes, fungal antagonists and *P. penetrans*.

Materials and Methods

An infestation of root-knot nematodes, a mixed population of *Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogyne javanica* (Treub) Chitwood, was observed in a tobacco field at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida. The severity of root-knot of tobacco had decreased over time at this site. *Pasteuria penetrans* was observed attached to second-stage juveniles (J2) extracted from soil collected from the site. Sampling was conducted in 1991 to determine the population densities of *Meloidogyne* spp. and nematode antagonists.

The site was planted to tobacco continuously for 6 years before 1991. The three $\times$ two $\times$ two factorial treatment design included: three autumn cover treatments, hairy indigo, forage sorghum, and weeds; two inorganic nitrogen fertilizers, 89 and 158 kg of ammonium nitrate/ha; and two tobacco cultivars, Coker 371 Gold and K-326. Coker 371 Gold is susceptible to both *M. incognita* and *M. javanica* and K-326 is resistant to *M. incognita* but susceptible to *M. javanica*. Hairy indigo and forage sorghum were planted in August and plowed under in November each year. Rye was planted in early December as a winter cover treatment over the entire field and plowed under in March. Tobacco was transplanted in late March, and the final harvest was taken at the end of

July each year. Preplant fertilization included a broadcast application of 1,800 kg of 6-6-18 (N-P-K) and 340 kg of 15-0-14 (sodium-potassium nitrate) per ha. At the final cultivation, in late April, ammonium nitrate was applied to the row middle at the rates described. The plots were arranged in randomized complete blocks replicated four times, and each plot consisted of one row with a row spacing of 1.2 m and length of 12 m.

Soil and root samples were taken with a bucket auger (10 cm diameter) from each plot in the root rhizosphere 25 cm deep on 4 June, 3 July, 25 July, and again on 23 August immediately after the soil had been disked. Six cores per plot were taken on 4 June, and 12 cores per plot were taken at all other dates. Soil from each sample, except for the final sample, was screened with a sieve with 4-mm openings to separate tobacco roots from the soil. The soil was mixed, and a 100-cm³ subsample was taken from each sample to extract nematodes by a centrifugal-flotation technique.²²⁵ The numbers of *Meloidogyne* J2/100 cm³ of soil were determined and the numbers of endospores attached to the cuticles of J2 were counted from 20 juveniles per sample with an inverted light microscope. Roots were washed and drained. Lateral roots were removed and chopped with a food blender for 20 seconds, put on tissue paper supported with a screen in a jar, and incubated at 23-24 C for 48 hours. The J2 were collected and counted.

On 2 August, six tobacco plants were removed from each plot and the galling indices were recorded according to the scale: 0 = no galls, 1 = 1-10, 2 = 11-20, 3 = 21-55, 4 = 56-80, and 5 = 81-100% roots galled.¹⁵ Twenty females were extracted from each root sample for estimation of the average number of endospores of *P. penetrans* per female. The females were ruptured and blended with a glass tissue grinder

in 2 ml of water and the *P. penetrans* endospores were transferred to a hemacytometer and counted. Ten egg masses were taken from each sample to determine frequency of colonization by fungi. The egg masses were washed three times with sterile water, treated with 0.5% NaOCl for 1 minute, washed with sterile water three times again, and treated with a solution containing 50 mg of chlortetracycline and 100 mg of streptomycin/liter of water for 10 minutes. The treated egg masses were transferred onto 1.5% water agar in petri dishes, five egg masses per dish. Following an incubation of 5 days at 23-24 C, the egg masses with and without fungi were recorded.

The density of nematodes and the numbers of *P. penetrans* endospores produced per female were transformed to $\log_{10} (x + 1)$ values. The numbers of *P. penetrans* endospores attached per J2 were transformed to $\log_{10} (100x + 1)$ values. Data that were calculated as percentages were transformed with $\arcsin (x)$. All data were subjected to analysis of variances (ANOVA). The means were compared with Duncan's multiple-range test or *t*-test when only one factor and two treatments were included. Linear regressions were performed to determine the relationship between the nematode density (transformed) or galling index (untransformed) with the nematode antagonists (transformed data).

Results and Discussion

The number of J2 in Coker 371 Gold plots was higher than that in plots planted with K-326 at all sampling dates ($P \leq 0.05$). No effect of inorganic nitrogen fertilizer or autumn cover treatments was observed on the population density of J2 in soil. All

three cultural practices affected the number of J2 hatched from roots ($P \leq 0.05$; Table A1-1). There was an interaction between the inorganic nitrogen fertilizer and autumn cover treatments affecting the numbers of J2 hatched from tobacco roots (Table A1-2). The high rate of ammonium nitrate suppressed J2 hatched from roots in plots of hairy indigo 4 June. In plots treated with the low rate of ammonium nitrate, a higher number of J2 hatched from tobacco roots from weed and hairy indigo plots than from forage sorghum plots. No similar effect was found in plots treated with the high rate of ammonium nitrate. In the weed plots, there was an interaction between cultivar and inorganic nitrogen fertilizer affecting the number of J2 hatched from roots 25 July. High levels of inorganic nitrogen decreased the number of J2 hatched from K-326 tobacco roots (Table A1-3).

The weed and hairy indigo plots yielded higher initial numbers of *P. penetrans* endospores attached to J2 and resulted in a higher percentage of J2 encumbered with *P. penetrans* endospores in samples collected on 4 June (Table A1-4). A lower percentage of J2 from Coker 371 Gold compared to K-326 was observed to have *P. penetrans* endospores attached in samples from 3 July. The high rate of ammonium nitrate decreased the percentage of J2 that had *P. penetrans* endospores attached in samples from 4 June (Table A1-4). There was an interaction between inorganic nitrogen fertilizer and cultivar affecting the number of *P. penetrans* endospore attached to J2 in soil 1 month after harvest (Table A1-5). *Meloidogyne* spp. females collected from tobacco grown in plots treated with the high rate of ammonium nitrate yielded higher numbers of

TABLE A1-1. Population densities of second-stage juveniles (J2) of *Meloidogyne* spp. in soil samples and the numbers of J2 hatched within 2 days from roots of two tobacco cultivars in a tobacco field treated with two inorganic nitrogen fertilizer rates and three autumn cover treatments.

Treatment	Level of treatment	J2/100 cm ³ of soil				J2/g of root		
		4 June	3 July	25 July	23 August	4 June	3 July	25 July
Nitrogen	89 kg N	169	866	1,584	190	161	234	134
	158 kg N	114	832	1,691	127	47	168	88
Cultivars	Coker 371 Gold	162	1,263	2,425	210	141	283	112
	K-326	121	434	850	107	66	120	110
Cover treatments	Weeds	135	961	1,689	234	82	239	123
	Hairy indigo	204	810	1,494	120	177	170	149
ANOVA	Forage sorghum	85	775	1,730	122	52	195	63
	Block	NS	NS	NS	NS	NS	NS	**
	Nitrogen (N)	NS	NS	NS	NS	*	NS	NS
	Cultivar (C)	*	***	***	***	***	***	NS
	Cover treatments(Ct)	NS	NS	NS	NS	NS	NS	**
	N × C	NS	NS	NS	NS	NS	NS	NS
	N × Ct	NS	NS	NS	NS	*	NS	NS
	C × Ct	NS	NS	NS	NS	NS	NS	NS
	N × C × Ct	NS	NS	NS	NS	NS	NS	*

Data are the means of main effects. The values were transformed to $\log_{10}(x + 1)$ values before being subjected to ANOVA. *, **, *** represent significant differences at $P \leq 0.05$, 0.01, 0.001, respectively. NS = not significant at $P \leq 0.05$.

Table A1-2. Interaction between nitrogen fertilizer levels and autumn cover treatments and their effects on the number of *Meloidogyne* spp. second-stage juveniles (J2) hatched over 2 days per gram of tobacco roots collected on 4 June.

Cover plant	J2/g of root	
	89 kg N	158 kg N
Weeds	131 aA	33 aA
Hairy indigo	314 aA	40 aB
Forage sorghum	38 bA	67 aA

The values were transformed to $\log_{10}(x + 1)$ values before being subjected to ANOVA and Duncan's multiple-range test. The same lowercase letter in columns or uppercase letter in rows indicate no significant differences at $P \leq 0.05$ according to Duncan's multiple-range test.

Table A1-3. Interaction between nitrogen fertilizer levels and tobacco cultivars and their effects on the number of *Meloidogyne* spp. second-stage juveniles hatched over 2 days per gram of tobacco root collected from weed plots on 25 July.

Cultivar	J2/gram of root	
	89 kg N	158 kg N
Coker 371 Gold	92 aA	138 aA
K-326	193 aA	67 aB

The values were transformed to $\log_{10}(x + 1)$ values before being subjected to *t*-test. The same lowercase letter in columns or uppercase letter in rows indicate no significant differences at $P \leq 0.05$ according to the *t*-test.

TABLE A1-4. Percentages of second-stage juveniles (J2) of *Meloidogyne* spp. attached with *Pasteuria penetrans* endospores, and average numbers of *P. penetrans* endospores attached per J2 in soil collected from two tobacco cultivars in a field treated with two nitrogen fertilizer rates and three autumn cover treatments.

Treatment	Level of treatment	Percentage of J2 with <i>P. penetrans</i> attached					Number of <i>P. penetrans</i> endospores attached per J2				
		4 June	3 July	25 July	23 August		4 June	3 July	25 July	23 August	
Nitrogen	89 kg N	55.6	51.3	71.5	39.6		3.5	3.2	10.1	1.4	
	158 kg N	46.3	46.9	73.8	36.9		3.2	2.7	8.9	3.2	
Cultivars	Coker 371 Gold	50.6	42.7	70.2	37.9		3.2	2.8	9.7	2.4	
	K-326	51.2	55.4	75.1	38.6		3.6	3.1	9.2	2.3	
Cover treatments	Weeds	57.5 a	47.8	75.3	41.6		3.8 a	1.7	10.6	2.7	
	Hairy indigo	53.1 a	51.3	77.0	41.0		4.1 a	3.9	11.0	3.1	
ANOVA	Forage sorghum	42.1 b	48.2	65.6	32.2		2.2 b	3.2	6.8	1.2	
	Block	**	NS	*	NS		NS	NS	*	NS	
Nitrogen (N)		*	NS	NS	NS		NS	NS	NS	NS	
	Cultivar (C)	NS	*	NS	NS		NS	NS	NS	NS	
Cover treatment (Ct)		**	NS	NS	NS		*	NS	NS	NS	
	N × C	NS	NS	NS	NS		NS	NS	NS	*	
N × Ct		NS	NS	NS	NS		NS	NS	NS	NS	
	C × Ct	NS	NS	NS	NS		NS	NS	NS	NS	
N × C × Ct		NS	NS	NS	NS		NS	NS	NS	NS	
		NS	NS	NS	NS		NS	NS	NS	NS	

Data are means of main effects. The percentages were transformed to arcsin (x) values and the average numbers of endospores per juvenile were transformed to $\log_{10}(100x + 1)$ values before being subjected to ANOVA. *, **, *** indicate significant differences at $P \leq 0.05$, 0.01, 0.001, respectively. NS = not significant at $P \leq 0.05$. Means followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple-range test.

Table A1-5. Interaction between inorganic nitrogen fertilizer levels and tobacco cultivars and their effects on numbers of *Pasteuria penetrans* endospores adhering to *Meloidogyne* spp. second-stage juveniles (J2) in soil 1 month after harvest of tobacco.

Cultivars	Endospores/J2	
	89 kg N	158 kg N
Coker 371 Gold	2.0 aA	2.7 aA
K-326	0.9 bB	3.8 aA

The values were transformed to $\log_{10}(100x + 1)$ values before being subjected to ANOVA. The same lowercase letter in columns or uppercase letter in rows indicate no significant differences at $P \leq 0.05$.

P. penetrans endospores than did females collected from plots treated with the low rate (Table A1-6).

It was shown that *P. penetrans* is suppressive to the root-knot nematodes. The population density of J2 in soil on 3 July was correlated negatively with both percentage of J2 with *P. penetrans* endospores attached ($r = -0.51$, $P \leq 0.001$), and average number of *P. penetrans* endospores attached to the J2 ($r = -0.33$, $P \leq 0.05$). A negative correlation ($r = -0.46$, $P \leq 0.01$) was also observed between the population density of J2 in soil on 25 July and the percentage of J2 in soil with *P. penetrans* endospores attached in the sample of 3 July. The number of J2 hatched from roots of the sample of 25 July was correlated negatively ($r = -0.57$, $P \leq 0.05$) with the number of *P. penetrans* endospores produced per female in K-326 plots previously covered with weeds or hairy indigo.

The frequency of fungal colonization of egg masses was higher on Coker 371 Gold than that on K-326, but no significant difference was observed between the two inorganic fertilizer levels or among the autumn cover treatments (Table A1-6). *Paecilomyces lilacinus* (Thom) Samson was the predominant fungal species encountered in egg masses of *Meloidogyne* spp. (data not shown); however, no significant correlation was observed between the density of nematodes and the frequency of fungal colonization of the egg masses.

Although significant changes in density of *Meloidogyne* spp. were observed at times with all three cultural practices, a difference ($P \leq 0.001$) in galling indices was observed only between the two cultivars (Table A1-6). A negative correlation ($r = -$

TABLE A1-6. Average numbers of *Pasteuria penetrans* endospores produced per *Meloidogyne* spp. female taken from roots of two tobacco cultivars, percentages of nematode egg masses colonized by fungi, and galling indices of tobacco roots grown in a field treated with two nitrogen fertilizer rates and three autumn cover treatments.

Treatment	Level of treatment	Number of endospores (10 ⁵ /female)	Percentage of egg masses colonized by fungi	Galling index
Nitrogen	89 kg N	1.15	46.4	3.4
	158 kg N	1.71	57.5	3.2
Cultivars	Coker 371 Gold	1.48	59.3	4.1
	K-326	1.38	44.6	2.5
Cover treatments	Weeds	1.15	52.8	3.4
	Hairy indigo	2.00	49.0	3.4
	Forage sorghum	1.14	54.1	3.0
	Block	NS	NS	NS
ANOVA	Nitrogen	*	NS	NS
	Cultivar	NS	*	***
	Cover treatment	NS	NS	NS

Data are the means of main effects. The average numbers of endospores per females were transformed to $\log_{10}(x + 1)$ values and the percentage of egg masses colonized by fungi were transformed to arcsin (x) values before being subjected to ANOVA. *, **, *** indicate significant differences at $P \leq 0.05$, 0.01, 0.001, respectively. NS = not significant at $P \leq 0.05$. No significant interaction among the factors was observed.

The galling index scale: 0 = no galls, 1 = 1-10, 2 = 11-20, 3 = 21-35, 4 = 36-50, 5 = 51-65, 6 = 66-80, 7 = 81-100% roots galled.

0.42, $P \leq 0.01$) was observed between the galling index of tobacco roots and percentage of J2 with *P. penetrans* endospores attached in soil 3 July. However, the overall density of *Meloidogyne* spp. in soil was relatively high in 1991. The above-ground symptoms associated with root-knot disease and root galling of tobacco were obvious in some plots, and a few plants died. The density of *P. penetrans* endospores is important for suppression of its host nematode population. Our results show some indications that higher nitrogen level increased the density of *P. penetrans* in the resistant tobacco cultivar. More studies are needed, however, before we can conclude whether or not these tactics could be used in IPM programs.

Summary

The response of a mixed population of *M. incognita* and *M. javanica* to three cultural practices, tobacco cultivars (two cultivars, differing in resistance to *M. incognita*), cover treatments (three treatments), and inorganic nitrogen fertilizer (two treatments), *P. penetrans*, and soil-borne fungi was investigated in a tobacco field in 1991. On all sampling dates, higher densities of root-knot nematodes were observed on tobacco cv. Coker 371 Gold than on K-326. Initially, forage sorghum, compared with weeds and hairy indigo, decreased the subsequent number of *Meloidogyne* spp. on tobacco, but had little effect on the density of second-stage juveniles (J2) in soil at mid-season or final harvest. The density of *P. penetrans* endospores also decreased in soil in forage sorghum plots. On one of four sampling dates, high levels of inorganic nitrogen fertilizer increased the number of *P. penetrans* endospores produced per

nematode female, and decreased the density of *Meloidogyne* spp. on tobacco. The density of *P. penetrans* endospores increased in these high-N plots after harvest, especially in the plots planted with K-326. The density of J2 in soil was correlated negatively with both the numbers of endospores attached per J2 and the proportion of J2 with attached endospores in midseason soil samples. No correlation was observed between the nematode density and frequency of fungi colonizing the egg masses of *Meloidogyne* spp.

APPENDIX 2

EFFECTS OF SOIL TREATMENTS ON THE SURVIVAL OF SOIL MICROORGANISM

Many physical and chemical means have been developed to eliminate or reduce population densities of soilborne plant pathogens for phytopathological studies.³⁴¹ Treatment of soil with fungicides, such as captafol,^{74,187} mancozeb and iprodione,³⁰⁹ benlate,³⁸³ tachigaren,³⁸³ and formalin,^{247,383} has been used in the evaluation of nematode suppressive soil. Microwave heating of soil may be useful to kill soilborne fungi with only little effect on prokaryotic organisms.¹³⁷ Subjecting soil to autoclaving or steam heating is also useful for killing soilborne fungi.³⁴¹

In the study of effects of soil mycoflora on the population of *Heterodera glycines* Ichinohe, treatments of soil by autoclaving, microwave heating, formalin, and storage at room temperature were used (Chapter 4). The effects of these treatments on survival of soil microorganisms are presented in this appendix.

Test 1. Soil was taken on 30 May 1992, with a bucket auger (10 cm in diameter) from six plots in the soybean field at the University of Florida Agronomy Farm, Green Acres, Alachua County, Florida. The soil type, texture, and chemical characteristics are summarized in Table 4-1 as soil 1 (Chapter 4). The soil was passed through a 6-mm-aperture sieve, mixed, and either treated with microwave heating¹³⁷ for 2, 3, 4, 5, or 6 minutes, or left untreated. For the microwave heating treatment, 1-kg

lots of soil were placed in even layers in open plastic bags and heated in a microwave oven (650 Watts) for the time required. The densities of prokaryotes and fungi in soil samples were determined by plating serial dilutions of soil suspensions in sterile water on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) and PDA containing 100 mg of streptomycin, 50 mg of chlortetracycline and 1.0 ml of tergitol/liter of medium,⁴⁴² respectively.

Test 2. Soil treated by microwave heating for 4 or 6 minutes, autoclaved, treated with formalin, stored at room temperature, or left untreated in experiment 1-3 in Chapter 4 was tested for survival of soil microorganisms following the treatments. The densities of prokaryotes and fungi were determined by following the procedure described above.

Results. Populations of the bacteria and fungi in soil decreased with the time of treatments with microwave heating ($P \leq 0.05$; Table A2-1 and Fig. A2-1). No bacterial colony was detected in soil treated for 6 minutes when the soil moisture was 5.2%. No fungus was detected in soil treated with microwave heating for 5 minutes at a soil moisture of 3.3% or for 4 minutes at 5.2%. No bacterial and fungal colonies were observed in autoclaved soil. The number of bacterial colonies increased 24 fold and the fungal colonies increased in soil following formalin treatment, as compared to untreated soil. The dominant fungal species encountered in the soil treated with formalin were *Trichoderma* spp. Storing soil at 23-24 C for 15 days had little effect on total bacterial and fungal densities (Table A2-1).

TABLE A2-1. Effects of soil treatments on survival of soil microorganisms.

Soil treatment		Original soil moisture (%)	Bacteria (10 ⁶ cfu /g of soil)†	Fungi (10 ⁴ cfu /g of soil)‡
Test 1				
Untreated		3.3	1.42	2.91
Microwave heating (minutes):	2	3.3	1.48	0.30
	3	3.3	0.38	0.09
	4	3.3	0.33	0.012
	5	3.3	0.021	0
	6	3.3	0.021	0
Test 2				
Untreated		5.2	1.68 b	1.93 c
Microwave heating (minutes):	4	5.2	0.022 c	0 d
	6	5.2	0 d	0 d
Autoclaving		5.2	0 d	0 d
Formalin treated for 15 days		5.2	41.67 a	6.82 a
Stored at 23-24 C for 15 days		5.2	0.91 b	2.56 b

Data are means of three replicates. The data were transformed to $\log_{10} (x + 1)$ values before subjecting to statistical analyses. In Test 1, the densities of bacteria or fungi are correlated negatively with time of exposure of the soil to microwave heating according to linear regression ($P \leq 0.05$). The same letters in columns in Test 2 indicate no significant differences at $P \leq 0.05$ according to Duncan's multiple-range test.

† Population in colony-forming units (cfu)/g of soil determined after incubation for 2 days on potato dextrose agar (PDA).

‡ Population in cfu/g of soil determined after incubation for 7 days on PDA containing 100 mg of streptomycin, 50 mg of chlortetracycline and 1 ml of tergitol/liter of medium.

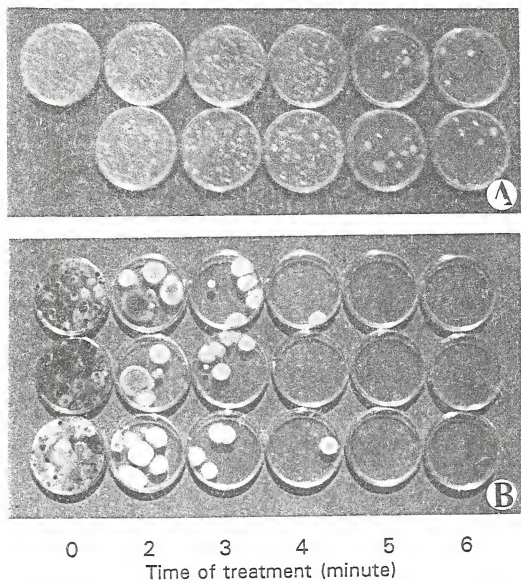


Fig. A2-1. Effects of soil treatments by microwave heating on survival of soil microorganisms. A) Bacterial colonies formed on potato dextrose agar (PDA) 1 week following the treatments. B) Fungal colonies formed on PDA containing 100 mg of streptomycin, 50 mg of chlortetracycline and 1 ml of tergitol/liter of medium 1 week following the treatments.

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BIOGRAPHICAL SKETCH

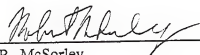
Senyu Chen was born on August 2, 1959, to Mrs. Maocai Huang and Mr. Shengyu Chen in Yueqing County, Zhejiang Province, the People's Republic of China. Senyu Chen received his elementary and middle school education from the local public school. He began his undergraduate education in the Department of Plant Protection of Zhejiang Agricultural University in November 1978, and received a Bachelor's degree in agricultural science in July, 1982. Nematology attracted him to the Plant Protection Institute, the Chinese Academy of Agricultural Sciences, where he worked in plant nematology for three years and obtained a Master of Science degree in November, 1985. He continued working in nematology, mainly on the soybean cyst nematode, in the Plant Protection Institute for the next five years. Chen came to the University of Florida in January, 1991, and has since worked in the laboratory of Dr. D. W. Dickson on biological control of plant-parasitic nematodes.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



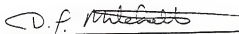
D. W. Dickson, Chair
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



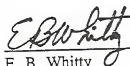
R. McSorley
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



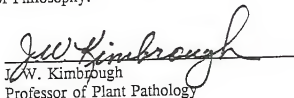
D. J. Mitchell
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



E. B. Whitty
Professor of Agronomy

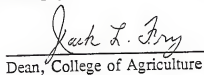
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



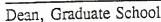
J. W. Kimbrough
Professor of Plant Pathology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1994



Dean, College of Agriculture



Dean, Graduate School